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University of Alberta

Genetic Studies of North American Wapiti Subspecies

by

Renee Otti Polziehn 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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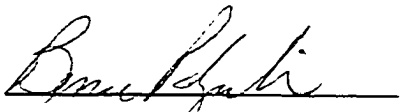

Renee O. Polziehn
11322-62 Street
Edmonton, AB
T5W 4C1

Date: October 2/2000

Abstract

Many species have seen changes to their classification since molecular information was made available. In this study, both mitochondrial (control region) and nuclear (microsatellite) loci were employed to support the divisions among subspecies of North American wapiti (*Cervus elaphus* ssp). The genetic markers were chosen on the basis that their higher rate of mutation would be needed to detect diversity among these highly related organisms. To put the observed genetic diversity among North American wapiti into perspective, the genetic differentiation was compared at a population, subspecies, and species level.

Overall, North American wapiti populations express little genetic variation, however isolated populations such as the Tule and Roosevelt wapiti were differentiated at both nuclear and mitochondrial markers. In contrast, the Rocky Mountain and Manitoban wapiti did not receive support for their subspecies designation from either set of markers. The translocation and migration of animals likely enhanced the similarity of genotypes among the Rocky Mountain and Manitoban wapiti. Their similar distribution of microsatellite genotypes and mitochondrial haplotypes suggests the separation among North American subspecies was recent.

When the comparison of mitochondrial DNA was extended to include Asian wapiti and European red deer, a division of haplotypes was detected along the Gobi Desert and Himalayan Mountains. The division of mitochondrial haplotypes corroborates the classification that recognized red deer as *Cervus elaphus* and wapiti as *C. canadensis*.

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitle Genetic Studies of North American Wapiti Subspecies submitted by Renee Otti Polziehn in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

A.T. Beckenbach

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Sean Graham

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Allen Good

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Table of Contents

List of Tables

List of Figures

Chapter 1

General Introduction

Rolston (1985) wrote, " destroying a species is like tearing pages out of an unread book, written in a language humans hardly know how to read." Given the rate at which human activity is having an impact on natural populations, there is an urgency not only to describe but also to understand existing species. Recent advances in molecular genetics have allowed species to be described beyond behavioral, morphological, and physiological observations. In addition, molecular tools have allowed comparisons to be made among incomplete specimens and have dramatically increased the number of homologous characters that can be compared. Furthermore, genetic characters unlike physical traits are not cloaked by the influences of sex, health, age, season, or environment (McHugh 1972; Berger & Peacock 1988; Geist 1991), and therefore they offer one of the most direct means of resolving relationships among individuals, populations, subspecies, and species.

In order to establish conservation and management plans for species, it is essential to determine the divisions that occur among organisms. In the case of the dusky seaside sparrow and tuatara (Daugherty *et al.* 1990; Zinc & Kale 1995), molecular comparisons have identified evolutionarily significant divisions among taxa that were overlooked using more classical methods of comparisons. Molecular characters also have been used to modify classifications where species were excessively 'split' by nineteenth century naturalists that had based their decisions on single type specimens (Dobzhansky 1951). The number of leopard subspecies for example was reduced from thirty-three to eight divisions using a combination of molecular and morphological traits (O'Brien 1994). Once species are suitably classified, conservationists face the difficult task of identifying populations that possess qualities necessary for the preservation of present and future generations (Ryder 1986).

Defining a species and subspecies

Because conservation efforts rely on species, subspecies, or population designations to varying extents, taxonomists, ecologists, and population geneticists are scrambling to identify populations they consider worthy of legislated protection. Defining a species or subspecies is challenging, as 1) no one definition can be applied to all organisms, 2) general knowledge about the ecology and diversity is lacking for many species, 3) habitat fragmentation and the extirpation of populations has created an artificial separation among remaining populations, and 4) hybridization events may blur the separation between populations.

Since the first explanations for the origin of species were put forward by the ancient Greeks (Futuyma 1986), the quest to define a species has produced more than 22 definitions (Mayden 1997). The most popular species definition applied to sexual diploid organisms is the Biological Species Concept, which as defined by Ernst Mayr in 1940, recognizes species as "groups of actually or potentially interbreeding populations that are reproductively isolated from other such groups." A variation of this definition is the Recognition Species Concept that requires members of a species to share a specific mate-recognition system.

Dobzhansky (1937) stated that "species are ... systems of populations [where] gene exchange between these systems is limited or prevented by a reproductive isolating mechanism or perhaps by a combination of several such mechanisms" although absolute reproductive isolation should not be used as the criterion of a species (Mayr 1940; Dobzhansky 1951). Several examples exist of species that are behaviorally, genetically and morphologically distinct but freely interbreed where they meet. The presence of such hybrids need not detract from the genetic integrity of a species because species commonly have adaptations to distinct niches and hybrids often have maladaptive gene combinations (O'Brien & Mayr 1991). Among cervids, Geist (1992) noted that fertile hybrid offspring were produced between sympatric populations of white-tailed deer and mule deer and also between sympatric populations of wapiti and sika deer. In both cases the offspring suffer from a hybridization of escape responses used by their parents (Geist 1992), which suggests hybrid viability may serve as another measure for a species designation as it demonstrates a level of reproductive isolation (Mayr 1940; Dobzhansky 1951).

Other species concepts include the Ecological Species Concept that defines a species as a set of organisms adapted to a particular ecological niche (Van Valen 1976), and the Cladistic Species Concept (Ridley 1989) that defines species by their phylogenetic relationships. In cladistic analysis, monophyly (all descendants of the most recent common ancestor are included in the group) and paraphyly (nearly all descendants of the most recent common ancestor are included in the group) is associated with the development of allopatric and parapatric species (Ashlock 1971). Species that are described as monophyletic or paraphyletic usually meet the requirements of most species definitions, while species that are described as a polyphyletic do not. Polyphyly is often based on convergent similarity (Hennig 1966; Ashlock 1971). Because polyphyly often results when hybridization occurs or when insufficient time has elapsed to allow lineage sorting (O'Brien & Mayr 1991), the most recent common ancestor of a polyphyletic species is not included in the species. If polyphyly is confirmed among species, the taxonomy is modified to reflect the most correct classification.

The latest species definition to gain attention, albeit a non-biological one, is the geopolitical species. Geopolitical species are defined as groups of individuals confined to geographically or politically defined areas and are accorded species status independent of morphological, genetic, or reproductive criteria (Karl & Bowen 1999).

Subspecies also can share many properties of species, however, they are best recognized when they show geographic partitioning. O'Brien and Mayr (1991) defined subspecies as "members [that] share: a unique geographic range or habitat, a group of phylogenetically concordant phenotypic characters, and a unique natural history relative to other subdivisions of the species." Avise (1989) and Ryder (1986) also proposed the subspecies designation should be based on phylogenetic relationships, with most subspecies being monophyletic because of the time-dependent accumulation of genetic differences in the absence of gene flow (O'Brien & Mayr 1991).

In addition to classical taxonomic units, evolutionary significant units (ESUs) and management units (MUs) can be used to describe organisms. ESUs recognize species, subspecies or populations that have a unique evolutionary potential, that should be reciprocally monophyletic for mtDNA alleles, and that show significant divergence of allele frequencies at a nuclear level (Ryder 1986; Waples 1991). ESUs differ from MUs that identify populations that diverge from one another at only nuclear or mitochondrial loci (Moritz 1994). Although the distinction between classical taxonomic units and ESUs is not entirely clear (Mayden 1997), the popularity of recognizing ESUs based on ecological and genetic data has increased acceptance for protecting biodiversity at a more local or population level (Crandall *et al.* 2000).

Genetics in general

The discovery of DNA and its various levels of organization have had a profound impact on our understanding of the diversity of life. The ability to answer questions relating to the genetic diversity of natural populations first appeared with karyotyping in the mid 1920's (White 1978) and improved with the refinement of protein electrophoresis in the 1960's (Avise 1994). Many studies today still rely on the analysis of proteins as a cheap and easy method of surveying large populations. Further variation in the DNA sequence was uncovered after restriction enzymes were isolated in the late 1960's (Meselson & Yuan 1968) and DNA sequencing was introduced in the 1970's (Maxam & Gilbert 1977; Sanger *et al.* 1977). Since the late 1980's, information gained from studies using the polymerase chain reaction (PCR, Mullis *et al.* 1986) has made available additional variation contained in nuclear and mitochondrial sequences of DNA.

Conservation issues that require information about an individual's contribution to the gene pool have also looked to DNA markers for answers. Following Jeffreys *et al.* (1985) discovery that genomes contain an abundant number of hypervariable regions, minisatellite probes were designed to detect repeats of 9 - 60 nucleotide units that allowed individuals to be identified. The discovery of microsatellite markers that consist of 1 – 6 repeating nucleotide units soon followed (Litt & Luty 1989; Tautz 1989; Weber & May 1989), and since their inception microsatellite markers have essentially replaced

minisatellite probes. Microsatellite markers are commonly used to survey the distribution of genetic variation and gene flow among natural populations.

Although mitochondria were known to possess their own DNA by the late 1960's, nearly a decade passed before population surveys appeared that used this resource of information (Avise *et al.* 1979; Brown *et al.* 1979). Shortly thereafter came the release of the first complete mitochondrial genome sequence (Anderson *et al.* 1981) and an interest in mitochondrial DNA that will likely continue well into the future, in particular for studies discussing evolutionary relationships.

Information gained from mitochondrial DNA (mtDNA)

Several properties of the mitochondrial genome make it useful for inferring relationships among vertebrates. The conserved arrangement of protein subunits, rRNA and tRNA genes allows homologous regions to be compared among fish, amphibians, and mammals (Wostenholme 1992). The primary mode of mtDNA inheritance is clonal and maternal in the egg, although examples of paternal inheritance have been reported (Avise 1991). Due to the absence of any DNA-repair mechanism or recombination (Grivell 1989), the mitochondrial genome is able to evolve quickly and lineages are not confounded by recombination events. The control region which includes the signals necessary for replication and transcription, has a 10 times greater mutation rate than single copy nuclear DNA (Brown *et al.* 1982) and may contain tandem repeat units that account for most large size differences among mtDNAs (Wostenholme 1992).

Most mitochondrial studies exploit sequences from the rRNA, protein subunits and control region to determine relationships among species and subspecies. However, complete mitochondrial genomes have been compared to determine relationships as distant as among orders (sequence data; Janke *et al.* 1997) and as close as kinships among populations (restriction enzymes; Kessler & Avise 1985). In combination with morphological data, mtDNA has been used to infer the process of speciation (Meyer *et al.* 1990; Zinc & Dittman 1993). The construction of mtDNA based phylogenies has also served to explain the evolution of behavioral and physiological adaptations (Clayton & Harvey 1993; Daltry *et al.* 1996) and the spatial and temporal distribution of species (Baker *et al.* 1990; Thomas *et al.* 1990; Norman *et al.* 1994).

Genetic relationships determined from mtDNA sequence differences are usually expressed in the form of a phylogeny, also called a gene tree, which identifies alleles as operational taxonomic units (OTUs; Avise 1989). A gene tree can vary from the true species tree for reasons such as an inadequate sample size, variation in the rate of mutation among lineages, stochastic sorting of ancestral lineages, and hybridization (Avise 1989). Although a species tree is best inferred from several independent gene trees, a mitochondrial gene tree has a better chance of agreeing with the species tree than any

single nuclear gene tree since it is based on smaller effective population size (Moore 1995).

The observed number of sequence differences also are used to estimate actual genetic distances among species, as well as to estimate the time that species diverged by assuming a molecular clock (Zuckerkandl & Pauling 1965). Caution is advised when employing the molecular clock for several reasons: base composition may not be stationary, rates of substitution can vary among genes, and rates of molecular evolution rely on external estimates of time (Moritz *et al.* 1987; Saccone 1994). For example, transitional mutations in the mitochondrial genome greatly outnumber transversional mutations particularly among closely related sequences of vertebrates, although as an artifact of saturation, the observed number of differences decreases as sequences diverge (Moritz *et al.* 1987). Fragment comparisons also may or may not be unreliable if there is length variation or if the overall sequence divergence exceeds 10 - 15 % (Moritz *et al.* 1987; Hillis 1998).

In summary, the general utility of mtDNA in elucidating mammalian phylogenies is related to its higher rate of mutation, the availability of known mtDNA sequences from many species, a higher likelihood of inferring the correct species tree, and the ability to estimate the time of divergence among organisms.

Wapiti as the species of interest

The North American elk or wapiti (*Cervus elaphus* subspecies), observed by European explorers in the 1500's, were likely named after the European elk (*Alces alces* or moose) due to a resemblance in size. Although "elk" is more widely used in North America, this common name has led to confusion at an international level, therefore the term wapiti will be used.

Although all organisms are worthy of scientific investigation, species that have considerable economic benefit, conservation interest, or unique biological systems usually offer the most practical choice of study organism because samples are usually easier to obtain and information is often available to aid in the interpretation of new data. Deer have been of economic interest to man for centuries, as can be seen from the trophy heads mounted in the royal European hunting lodges, antler extracts used in ancient medicinal tinctures, and the use of wapiti teeth as currency among native Americans. Although the dependence of man on wapiti as a source of clothing or food has declined, these majestic animals continue to represent an image of true wilderness to people. Maintaining populations is a requirement not only for tourists and hunters, but also for large carnivores that rely on wapiti as an important food source. Therefore wildlife managers must monitor wapiti populations to ensure healthy animals will continue to exist in the future.

Currently, a purpose for determining the taxonomic classification of species is to provide protection under conservation programs. Conservation efforts are usually directed

to animals classified as endangered or threatened and at more than a million animals, the North American wapiti does not come to mind as a group requiring protection. However, North American subspecies comprise only 4 of the 23 extant *Cervus elaphus* subspecies distributed across the northern hemisphere, and a comparison of their genetic differences may identify significant evolutionary relationships that may not be recognized by the current taxonomy. To develop a conservation plan for *Cervus elaphus*, genetic information will be required from a population to a species level.

On a larger scale, patterns of sequence divergence among species may help explain the current distribution and diversity of large land animals in North America, Asia, and Europe. Wapiti are one of several species to disperse from Asia into North America and related populations can still be found in Asia and Europe. Although accessibility to samples from Asian animals listed on the CITES Appendix is restricted, the ability to isolate DNA from hair samples has made it possible to obtain DNA from rare animals. In contrast, tissue samples from North America are in abundant supply from hunted and road-killed animals.

Taxonomy of *Cervus elaphus*

When a species has a wide distribution, such as red deer (*Cervus elaphus* Linnaeus - 1758), there is often little consensus as to whether adjacent populations and subspecies are synonomous. Supporters of the opinion that *C. elaphus* should be separated into red deer and wapiti species were found as early as 1737 (Bryant & Maser 1982), and they referred to the wapiti group as *C. canadensis* -Borowski 1780 (Hall & Kelson 1959; Whitehead 1972; Cockerill 1984). However, the phenotypic similarity between red deer and wapiti led Ellerman and Morrison-Scott (1951) and Flerov (1952) to consider wapiti from North American and European red deer as conspecific, and currently all wapiti are regarded as subspecies of *C. elaphus* Erxleben (1777).

Although the reigning opinion recognizes three divisions among red deer that correspond to regions of Europe, Central Asia, and Eastern Asia/America (Groves & Grubb 1987), an alternative division recognizes a separation between red deer and wapiti that corresponds to the geographic division formed by the Himalayan mountains and Gobi desert (Cockerill 1986). Lowe and Gardiner (1989) suggested that the gap between Old World red deer and New World wapiti was more than just geographic, but conceded that behavioral and reproductive differences were not diverged enough for New World animals to be elevated to a species level.

The divisions among wapiti subspecies within North America have also been debated. Currently recognized are the extant Rocky Mountain (*C. e. nelsoni* Bailey 1935), Manitoban (*C. e. manitobensis* Millais 1915), Roosevelt (*C.e. roosevelti* Merriam 1897), and Tule subspecies (*C. e. nannodes* Merriam 1905), and the extinct Eastern (*C. e. canadensis* Erxleben 1777) and Merriam subspecies (*C. e. merriami* Nelson 1902). The

similarity among North American subspecies had led to Rocky Mountain, Manitoban, Eastern, and Roosevelt wapiti collectively being known as *C. e. canadensis* (Flerov 1952; Hall & Kelson 1959).

Genetic Comparisons Among *Cervus elaphus*

Genetic variation among cervids has been explored using chromosomes, allozymes, and mitochondrial DNA. Interfamilial (Wessman & Gripenberg 1993) and interspecific karyotype variation was noted among Cervidae and admixtures of chromosomes were found when species hybridized, yet no diagnostic features could be used to discriminate red deer and wapiti (Bartos & Zirovnicky 1982; Fontana & Rubini 1990; Herzog & Harrington 1991). Initial allozyme studies noted little genetic variation among proteins of large mammals (McDougall & Lowe 1968; Ryman *et al.* 1980; Baccus *et al.* 1983; Wooten & Smith 1984), although the technique is commonly used to estimate diversity within and among red deer populations (Van Wieren 1989; Herzog *et al.* 1991; Strandgaard & Simonsen 1993; Hartl *et al.* 1995). Marked differences were noted among proteins of red deer and wapiti (Dratch & Gyllensten 1985; Baccus *et al.* 1983), and the Dratch test that identifies serum proteins remains in use today as a means of identifying red deer-wapiti hybrids (Dratch 1986). When protein electrophoretic studies were used to determine the variation among North American populations, Rocky Mountain and Tule wapiti were shown to be genetically depauperate and populations were indistinguishable (Cameron & Vyse 1978; Kucera 1991; Glenn & Smith 1993). Although mitochondrial DNA (mtDNA) has proved to be suitable for large population surveys, only two studies (Cronin 1992; Murray *et al.* 1995) in addition to my own work suggest that variation can be found among mtDNA of wapiti subspecies. The possibility that sika deer were also part of a red deer-wapiti cline was explored using allozyme data and differences were again noted among the three groups (McDougall & Lowe 1968; Emerson & Tate 1993).

Among *Cervus* species minisatellite and microsatellite studies have primarily focussed on determining paternity (Pemberton *et al.* 1995; Talbot *et al.* 1996). Three exceptions are the application of mtDNA and microsatellite markers by Goodman *et al.* (1999) and Abernathy (1994) to demonstrate the changing sika-red deer hybrid zone in Britain and the application of Jeffrey's probes to show the disappearance of genetic variation in isolated and reintroduced populations of Spanish red deer by Perez *et al.* (1998).

Genetic research involving the relationships among cervids subfamilies was also pursued using mtDNA (Miyamoto *et al.* 1990; Cronin 1991; Douzery & Randi 1997), satellite DNA (Lima-de Faria *et al.* 1984; Bogenberger *et al.* 1987; Schertharn *et al.* 1990; Lee *et al.* 1997) and RAPDs (Comincini *et al.* 1996). Unfortunately, no comprehensive study exists that includes all members of each cervid subfamily; therefore little has been

gained in the understanding of the evolution among species of the Cervidae. In addition, the phylogenetic relationships among the Cervidae remain suspect because species such as the moose, caribou, and Chinese water deer have no close living relatives, which are required to infer the most likely ancestral states.

Objectives of Project

Although mtDNA phylogenies are abundant, few studies include all subspecies that cover the range of a species. The focus of this project was to examine the genetic diversity observed within North American subspecies of wapiti, and to determine whether the variation in genetic differences would corroborate the current classification of *Cervus elaphus*. The genetic studies described in this thesis are divided into variation within and among subspecies. The chapters presented in this thesis move from a population level to a species level, which is contrary to the order they were discussed throughout the introduction. Although species concepts are more easily described from a species to a population level, measures of genetic variation are more easily presented from a population to a species level. The increase in the amount of genetic diversity as comparisons progress from a population to a species level was used in this study to support the classification of the *Cervus* subspecies.

Genetic variation within Subspecies

A species' pool of genetic diversity exits at three fundamental levels: variation within individuals, among individuals, and between populations, where each level is a genetic resource of potential importance to conservation, so each must be understood relative to the others. The application of microsatellite markers to differentiate populations and mtDNA markers to follow lineages are discussed in Chapters 2 and 3, with the purpose of determining factors that affect genetic variation within and among populations of four North American subspecies. Populations that demonstrate a restriction to gene flow by their distinct allele frequencies and mtDNA signatures show strong support for subspecies designations. The combination of mitochondrial and nuclear markers is useful because their different rates of mutations are sensitive to different levels of genetic differentiation and both are necessary when the populations in question have different histories of isolation. The information presented in Chapters 2 and 3 provides wildlife biologists with an overview of the genetic diversity among wapiti populations so they may consider at what level their management actions will affect these populations.

Genetic variation among subspecies

Genetic differentiation and phylogenetic relationships were utilized to classify subspecies of *C. elaphus*, however, defining subspecies based on their phylogenies has proven challenging for many species. The controversy surrounding the evolutionary divisions of species, such as brown bears (Paetkau *et al.* 1998), sea turtles (Karl & Bowen 1999), and red wolves (Nowack 1991) involves an incompatibility of phylogenies produced from molecular and traditional methods of classification. The role of mtDNA in determining the taxonomic division between red deer and wapiti is discussed in Chapters 4 and 5. A preliminary study described in Chapter 4 highlights the significant amount of variation in the control region of mtDNA between red deer and wapiti, and among the higher taxonomic divisions of the family Cervidae. Since the level of variation detected between the control region of red deer and wapiti mtDNA suggested wapiti and red deer each be raised to a species level, the project was extended to include samples of subspecies that would represent the entire *C. elaphus* distribution. The phylogenetic relationships among wapiti and red deer explored in Chapter 5 evaluated whether *Cervus elaphus* subspecies should be recognized as a continuum of phenotypic variation or whether they should be divided into two species.

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Chapter 2

Phylogenetic Status of North American Wapiti Subspecies (Cervus elaphus spp)[1]

Abstract

By the turn of the century, North American elk or wapiti (*Cervus elaphus)* had been extirpated from a range of populations across all regions of the continent and two subspecies were extinct. The recovery of wapiti is largely a response to the large number of relocated Rocky Mountain (*C. e. nelsoni*) and Manitoban wapiti (*C. e. manitobensis*). A phylogenetic study was performed to determine the present genetic relationships among Tule (*C. e. nannodes*), Roosevelt (*C. e. roosevelti*), Rocky Mountain, and Manitoban subspecies, using sequences from the D-loop region of mitochondrial DNA of 28 individuals. All Roosevelt wapiti were grouped together, as were the Tule wapiti, which supports the classification of these subspecies. Yellowstone, Elk Island, and Riding Mountain National Parks have not introduced wapiti into their indigenous populations. When these populations were used, Manitoban wapiti were found to be monophyletic and Rocky Mountain wapiti to be paraphyletic. However, including animals from the Canadian Rocky Mountains places Rocky Mountain wapiti in clades by themselves or grouped together with Manitoban wapiti. The clade containing a mixture of Manitoban and Rocky Mountain wapiti suggests that both types recently descended from a common ancestor. Hybridization or insufficient time for separation may explain the presence of both types in the same clade.

Introduction

Herds of North American elk (*Cervus elaphus*), also known as wapiti recently inhabited nearly every region of North America. Wapiti populations were tenuously classified into six subspecies that corresponded to their biogeographical distribution and ecozones (Bryant and Maser 1982). The classification of wapiti has been examined using morphology, behavior, and more recently molecular characteristics (Bryant and Maser 1982; Cronin 1992). However, little consensus exists regarding the subspecies distinctions. Determining evolutionary relationships among taxa can assist the

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conservation and management of species. Populations that have been historically isolated and likely to possess a unique evolutionary potential are called Evolutionary Significant Units (ESU, Moritz 1994). ESU's should be monophyletic for mtDNA and show significant divergence of allele frequencies at nuclear loci (Moritz 1994). In this phylogenetic study, mitochondrial DNA (mtDNA) was employed to determine the validity of North American wapiti subspecies.

Presently, six subspecies of wapiti are recognized in North America, including the extant Manitoban (*C.e. manitobensis* Millais 1915), Rocky Mountain (*C. e. nelsoni* Bailey 1935), Roosevelt (*C.e. roosevelti* Merriam 1897), and Tule wapiti (*C. e. nannodes* Merriam 1905) and the extinct eastern (*C. e. canadensis* Erxleben 1777) and Merriam (*C.e. merriami* Nelson 1902) wapiti. Earlier classifications of wapiti, however, considered North American animals distinct from the European red deer and also had fewer divisions. This is demonstrated by Murie (1951) who accepted only two species of North American wapiti: *C. canadensis* and *C. nannodes*. *C. e. canadensis* was used to describe the eastern, Rocky Mountain, Manitoban, and even Roosevelt wapiti (Bryant and Maser 1982). Currently, subspecies found in adjoining ranges are still considered by some as one entity. Schonewald (1994) suggested the extinct Merriam wapiti was an extension of the Rocky Mountain type.

Post glacial distributions for the various forms of wapiti (see Figure 2-1) have been discussed by Geist (1971), Banfield (1974), Bryant and Maser (1982), and Peek (1982). Historically, the Rocky Mountain wapiti range followed the Rocky Mountains and extended across the northern Canadian boreal coniferous forest. The Manitoban wapiti range covered the region of the prairies known as the Great Plains. The Eastern wapiti range corresponded with the eastern deciduous forests that lay parallel to the Manitoban wapiti range and the Atlantic coast, with a northern limit of the Great Lakes and a southern limit of northern Florida. The Merriam wapiti range occurred south of the Rocky Mountain wapiti range and covered the states of Arizona, Texas, New Mexico, and Mexico. The Roosevelt wapiti range extended along the west coast from southern British Columbia to northern California, while the Tule wapiti range was enclosed by the Sierra Nevada-Cascade Mountains in southern and central California (Bryant and Maser 1982).

Hunting and ranching activities led to the extirpation of wapiti from most of their native ranges, and by 1900 only a few herds were found in North America. The Tule animals (reports range from one pair to 100) were salvaged by Henry Miller during the mid 1870's and given refuge on his ranch in California (Bryant and Maser 1982). The Merriam wapiti was thought to have gone extinct at the start of the 1900's and the last Eastern wapiti was seen in 1893 near North Bay, Ontario (Bryant and Maser 1982).

Small herds of Roosevelt wapiti survived on Vancouver Island, British Columbia; on the Olympic Peninsula of Washington; and in the Cascade Mountains of Oregon.

The difficult terrain in British Columbia provided refuge for several (10 - 20) isolated herds of Rocky Mountain wapiti (Spalding 1992). In Alberta, these wapiti were reduced to a few dozen in the Brazeau and Highwood River drainages, and approximately 150-300 in the Old Man River drainage (Bryant and Maser 1982). Wapiti were never common in the valleys of Jasper or Banff National Park (Kay *et al.* 1994). Legislated protection and inhospitable terrain also contributed to the survival of Rocky Mountain wapiti in Colorado, Montana, and Wyoming. The largest herd (>1000 animals) to survive the great extirpation was found in Yellowstone National Park (Houston 1974).

Manitoban wapiti, abundant throughout Alberta until 1810, were reduced to 24 animals in Elk Island National Park by 1906 (Blyth and Hudson 1987), and an unknown number of animals were thought to have existed in the Cypress Hills. Few animals survived in the open prairies and no Manitoban wapiti were found in the United States after 1900. The largest concentration of Manitoban wapiti was found in Riding Mountain National Park, Manitoba, which began with more than 500 animals (Banfield 1949).

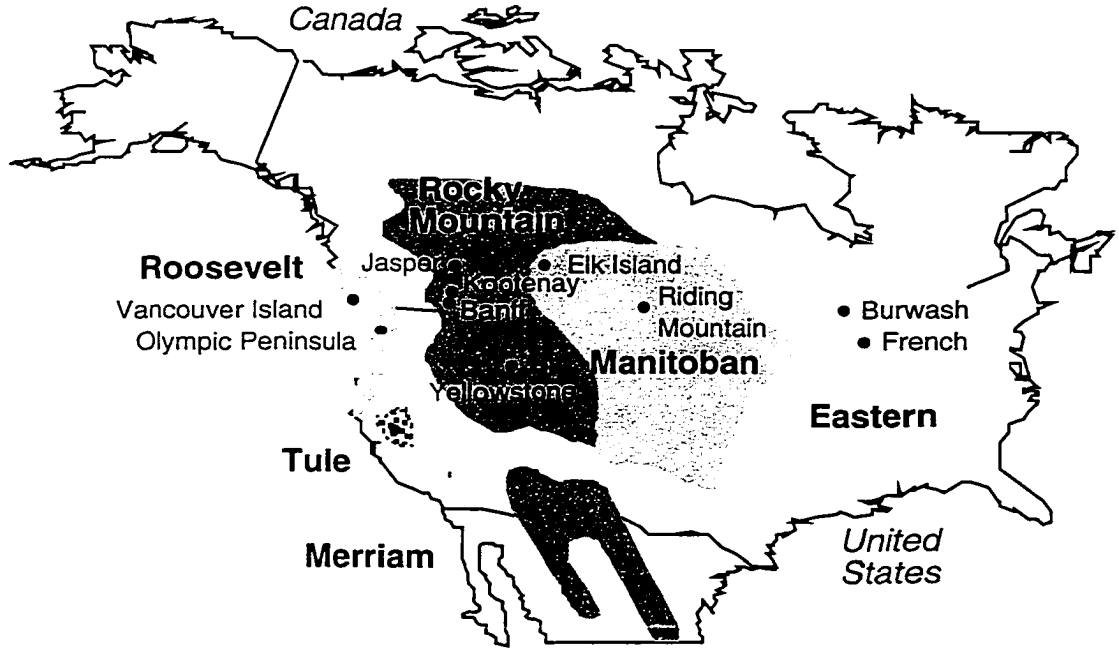


Figure 2-1. The historical ranges of the Roosevelt, Rocky Mountain, Manitoban, Eastern, Merriam, and Tule wapiti, adapted from Bryant and Maser (1982). Identified are the sample locations of each of the four extant and one extinct subspecies used in this study.

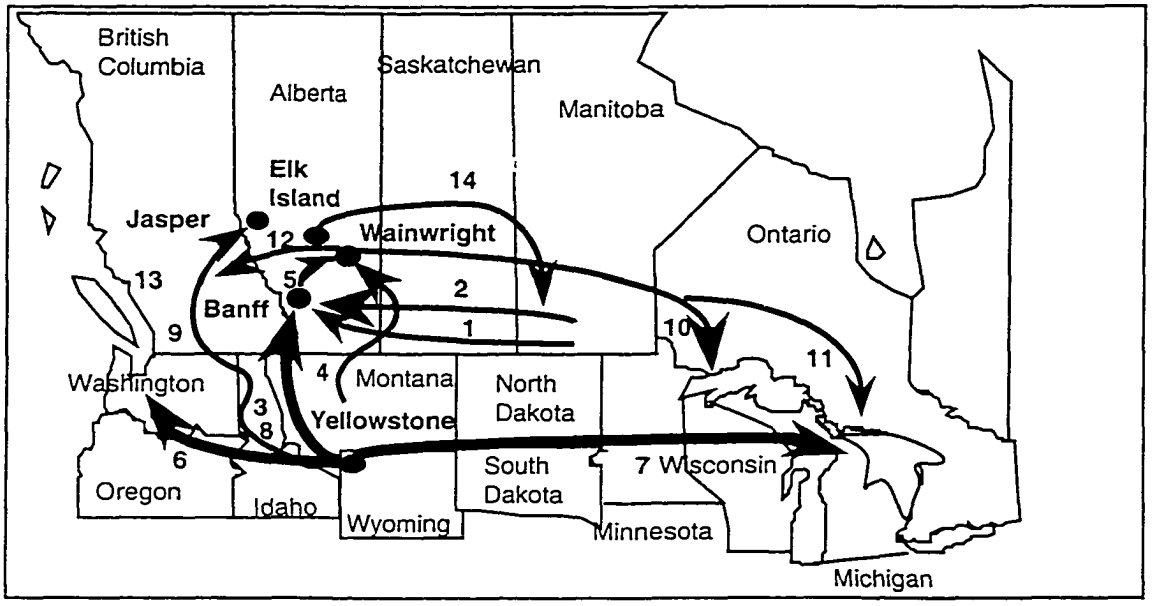
The similar appearance of wapiti in the different ranges led to questions regarding their taxonomic status. However, morphological comparisons failed to find unique or indisputable characters that can discriminate between the different wapiti subspecies. Skull and antler characters both separated (McCullough 1969; Hutton 1972) and lumped together (Green 1956; Blood and Lovaas 1966; Hutton 1972) subspecies. The Manitoban wapiti were described as both smaller (Soper 1946) and larger (Blood and Lovaas 1966) than the Rocky Mountain wapiti. However, there is little dispute that the Tule form is both smaller and lighter in coat color than other forms. As well, the Roosevelt form tends to be larger and heavier in stature than Rocky Mountain form, with more massive but shorter [crownlike] antlers, shorter tails, longer hind feet, and greater contrast between light and dark portions of the coat (Schwartz and Mitchell 1945; Quimby and Johnson 1951).

Morphological characters are encoded by DNA, but are influenced by age, sex, and health of an animal, as well as by seasonal and habitat conditions (Berger and Peacock 1988; McHugh 1972; Geist 1991). Comparisons of DNA removes these complex influences yet allowing one to still use characters that are under evolutionary constraints. Few studies have been directed at identifying the diversity of wapiti. Chromosome numbers vary within the genus *Cervus* (Fontana and Rubini 1990), but are constant among North American wapiti subspecies. Hemoglobin (Dratch 1986) and protein electrophoresis studies (Dratch and Gyllensten 1985) identified loci that were both unique and fixed in either red deer or wapiti, but they did not separate North American animals into subspecies. Glenn and Smith (1993) failed to differentiate among five of seven Rocky Mountain populations using protein variation. They did note the amount of polymorphic loci (P) was 0.087 in wapiti with an average of 1.1 allele per locus and that there was a slight distinction between Roosevelt and Rocky Mountain populations. A lack of variation was also observed by Cameron and Vyse (1978) who found P = 0.0416 in Yellowstone National Park, and by Kucera (1991) who observed P = 0.053 in the Tule wapiti. RAPD analysis of wapiti suggested the similarity among individuals ranged from 0.976 to 0.947 (Comincini *et al.* 1996).

Clearly, genetic variation exists in wapiti populations, albeit reduced. DNA regions that have highly evolving sequences, such as the D-loop region of mitochondrial DNA (mtDNA) will usually produce more variable characters and, therefore they are best suited for distinguishing among closely related taxa. Total mtDNA analysis using restriction enzymes failed to uncover unique differences among 22 wapiti (Cronin 1991). While the restriction analysis of the wapiti by Cronin (1991) or Murray *et al.* (1995) assayed at most a few hundred nucleotides, while sequencing would have assayed thousands. In a study to determine genetic variation between subspecies, Cronin (1992) found one unique

haplotype in the Rocky Mountain population. In addition to the common haplotype found among the Rocky Mountain and Manitoban animals, restriction analysis of the D-loop region of mtDNA from 59 wapiti by Polziehn (1993) and Murray *et al.* (1995) confirmed a unique *Cfo I* restriction pattern for 15.8% (3/19) of Rocky Mountain wapiti and a *Hinf I* restriction site for all 25 Roosevelt wapiti.

Figure 2-2. Relocations of wapiti in North America (Lloyd 1927: Bryant and Maser 1982; Stelfox and Stelfox 1993). Only a few of the hundreds of introductions that have taken place since 1900 are shown. These transfers illustrate the potential for hybridization to occur between subspecies.



Event	Date	Description
1	1900	Banff National Park received four bulls and one cow from Mrs. Ticknor of Morden, MB.
2	1902	Banff National Park received one cow from Portage la Prairie, Manitoba (and one cow from Calgary, Alberta).
3	1910	Banff National Park purchased two cows and two bulls originating from Wyoming.
4	1910	Wainwright Buffalo Park purchased two bulls and one cow from Montana.
5	1910	Wainwright Buffalo Park received six wapiti from Banff National Park.
6	1913	Yellowstone National park shipped wapiti for 20 years into the Selkirk and Wenatchee Mts.
7	1915	Yellowstone National park introduced 23 animals to Sturgeon County, Michigan.
8	1916 1920.	Yellowstone National Park shipped 66 wapiti to Banff National Park and another 196 in
9	1920	98 Yellowstone National Park wapiti were introduced into Jasper National Park.
10	1930s	24 wapiti from Wainwright Buffalo Park were introduced to the Burwash Industrial Prison near Sudbury, Ontario.
11	1920 - 1940	Wainwright Buffalo Park sent wapiti to the Nipigon-Onamon Game Preserve and an enclosure near Pemberton, Ont. Animals from the enclosure were relocated to the Bruce Peninsula, Abitibi, Peterborough, and Marten River.
12	1927	Wainwright Buffalo Park shipped 25 wapiti to Cookson, B.C.; in 1933 another 25 animals went to Adams Lake, British Columbia.
13	1936	Yellowstone National Park wapiti shipped to Hinton, Alberta near Jasper National Park.
14	1949	Elk Island National Park shipped an unknown number of animals to The Pas, Manitoba.

The relationship between genetic and geographic distribution has been used to augment classical taxonomy. However, employing genetic diversity to identify wapiti subspecies and their range has been complicated by numerous relocations of animals. Transplanting wapiti throughout North America gained popularity when populations started flourishing in Yellowstone National Park, Olympic Peninsula National Park, Elk Island National Park, Riding Mountain National Park, and on private lands in California. Relocations with significance to this study are listed in Figure 2-2. Many past introductions have moved wapiti from one subspecies into the range of another subspecies, and remarkably, similar events still occur. For example, in 1984, a group of Manitoban wapiti was released in the Kechikan River Valley, home of a native herd in coastal British Columbia.

A phylogenetic analysis of the D-loop region of mtDNA was performed to investigate genetic variability among wapiti and to determine if the genetic relationships correspond to the modern distribution of wapiti subspecies. When all descendants from the most recent common ancestor were found to belong to one subspecies, the subspecies is called monophyletic. Monophyletic groups provide strong support for subspecific status. Paraphyly occurs when not all descendents of the most recent common ancestor are found in one subspecies. Paraphyly can occur among well defined subspecies. Subspecies that arose from several recent common ancestors or lineages are called polyphyletic. Polyphyly is usually apparent when there has been insufficient time for populations to become distinct, or occurs as a consequence of hybridization or relocation. Evidence of polyphyly in such groups argues against the biological reality of a subspecies.

Materials and Methods

Collection

Samples representing Rocky Mountain wapiti were collected opportunistically from the following National Parks: Jasper (91 and 92), AB; Banff (14, 23, and 37), AB; Kootenay (KNP), BC and Yellowstone (1 and 2), WY. Samples from Manitoban wapiti were collected from animals restrained for transport from Elk Island National Park (EINP), AB, and opportunistically from animals from Riding Mountain National Park (RMNP), MB. Samples potentially representing *C. e. canadensis* were also collected from the French (Ont B5A and Ont B6) and Burwash (Ont T1 and Ont T5(55) River regions south of Sudbury, Ontario. Roosevelt samples (Roos 23, 25, 29, 32, and 33) were supplied from the Alberta and British Columbia Fish and Wildlife Services. The Forensics Laboratory of the US Fish and Wildlife supplied lyophilized samples from sika

deer (215 and 226), red deer (765 and 923), Tule wapiti (457 and 659) and Roosevelt wapiti (10 samples). The location of the samples are marked on Figure 2-1.

Isolation and Amplification (polymerase chain reaction)

DNA was isolated as in Bork *et al.* (1991) or using methods described in the Qiagen QIAamp tissue isolation kit (Chatsworth, CA). The D-loop region of mtDNA was enzymatically amplified in 100 μL reaction containing 0.06 mM each of dATP, dCTP, dGTP, and dTTP, 1X PCR buffer (10 mM Tris buffer, pH 8.8, 0.1% Triton X100, 50 mM KCl, and 0.16mg/mL BSA), 1 unit of *Taq* polymerase, 2.0 mM magnesium chloride, and 20 pmol each of primer CST 2 and 39 (Table 2-1). Primer CST 2 anneals to the start of the tRNA proline gene upstream from the D-loop region, and CST 39 anneals to the start of the 12S gene, downstream from the tRNA phenylalanine gene and D-loop region. These primers were based on 'universal' D-loop primers described by Kocher *et al.* (1989). Each 100 μL amplification reaction was performed on a 9600 Perkin Elmer Cetus thermocycler using the following conditions: a 3 min. denaturing step at 94 °C; 30 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 30 s; with a final 10-min. extension at 72 °C. The amplified products were separated from unincorporated primers by electrophoresis on a 1% agarose 0.5 x TBE gel. DNA fragments containing the D-loop region were excised from the gel with a scalpel and the DNA was isolated using a Qiagen Qiaquick extraction kit. Samples were desiccated and resuspended in 36 μl double-distilled water.

Table 2-1. Primers employed in the sequencing of the control region of mitochondrial DNA in cervids. Primers indicated with an asterisk * were published in Polziehn (1993).

Primer (CST)	Location	Sequence (5' - 3')
2*	1-22	TAATATACTGGTCTTGTAAACC
25*	614-591	TCATGGGCCGGAGCGAGAAGAGG
39*	1216-1192	GGGTCGGAAGGCTGGGACCAAACC
139*	493-522	ATGTCAAATCTACCCTTGGCAACATGCGTA
149*	763-730	AGCACAGTTATGTGAGCATGGGCTGATTGG
463	714-733	CTCGATGGACTAATGACTAA
464	275-294	CTCGTAGTACATAAAATCAA
468	990-968	ATAAGGGGGAAAAATAAGAA

Each sequencing reaction of the D-loop region was performed using 8 μL purified PCR product, as described in the Perkin Elmer Dye Terminator Cycle Sequencing Ready Reaction kit. Primers used for sequencing are given in Table 2-1. Cycle sequencing reaction parameters on the 9600 Perkin Elmer Cetus thermocycler were: denaturation at 96 °C for 15 s, annealing at 50 °C for 1 s, and extension at 60 °C for 4 min. Sequencing reactions were separated by electrophoresis on an ABI Prism 377 Perkin Elmer automated

sequencer. Sequence data was processed and analyzed using ABI sequence software.

PCR products from the ten Roosevelt samples from the Olympic Peninsula were cut with the endonuclease *Hinf I.* This enzyme was thought to distinguish Roosevelt wapiti in the D-loop region of mtDNA from other wapiti subspecies. The fragments were separated by electrophoresis on a vertical gel apparatus as described by Murray *et al.* (1995).

Phylogenetic Analysis

Once sequences were aligned using software Sequence Editor™ (Applied Biosystems), nucleotide substitutions, deletions, and insertions were identified. Sequences were analyzed using a heuristic search for their phylogenetic content using general search options of PAUP 3.1 (Swofford 1993). The PAUP program constructs phylogenetic trees based on parsimony criteria. Trees were constructed using equally and unequally weighted characters, where transversions were worth 2, 5, and 10 times more than transitions and gaps were equally weighted to transitions. Gaps generally occurred in tandom repeats of a single nucleotide. Trees were rooted using red deer and sika deer, and examined for polyphyly. Bootstrapping was used to place confidence estimates on branches within the most parsimonious trees and was restricted to 100 replicates. Trees were constructed for pure populations, as well as for populations known to have introductions.

Divergence

The following estimates of DNA divergence are taken from Nei (1987). The average number of nucleotide substitutions for haplotypes (d_X) in population (X) are estimated by $dx = \frac{n_x}{n_x - 1} \sum_{ij} x_i x_j d_{ij}$, where n_X is the number of sequences sampled and d_{ij} is the number of nucleotide substitutions per site between the i^{th} and j^{th} haplotypes. The average number (d_{XY}) of nucleotide substitutions between DNA haplotype from populations X and Y are estimated from $dxy = \sum_{ij} x_i y_j d_{ij}$, where d_{ij} is the number of substitutions between the i^{th} haplotype from X and j^{th} haplotype from Y. The number of net nucleotide substitutions can be estimated by substracting the average intra-population distance from the intra-population distance, given as $\pi = dxy - (dx + dy) / 2)$.

Results

The D-loop region of mitochondrial DNA amplified from the four subspecies of the North American wapiti was 1211 base pair (bp), in comparison with 1135 bp for red

deer and 1215 bp for sika deer. Compared to the North American wapiti, red deer had four insertions, and three deletions with one deletion of 77 bp (Table 2-2). Sika deer had four insertions relative to the North American wapiti.

In addition to two red deer and two sika deer sequences, 25 unique sequences were recognized from the 28 wapiti analyzed. Sequences submitted to GenBank have accession numbers AF005196-5200, AF016953-16977, and AF016979-16980. In the phylogenetic analysis, there were 40 variable nucleotide sites among the wapiti sequences (Table 2-3), including 27 transitions, 9 tranversions, two insertions, and two deletions. There were 17 uniformative sites (113, 315, 476, 541, 681, 709, 798, 838, 852, 935, 942, 951, 986, 1025, 1054, 1117, and 1138) and 23 informative sites (181, 269, 440, 442, 444, 448, 450, 487, 488, 493, 627, 679, 694, 703, 717, 737, 808, 867, 960, 968, 981, 988, and 1154). Sites 440, 442, 448, 487, 488, 627, 679, 694, and 703 were homoplastic between wapiti and the out-groups, and characters 181, 450, 486 or 968, 960, and 988 were homoplastic among only wapiti. Characters at sites 181, 960, 968, and 981 represent the absence/presence of a nucleotide in a long repeat of the identical nucleotide. Similarly, nucleotide substitutions found within a string of repeats include characters at sites 694, 709, 867, 951, and 1154. Replication errors are more likely to occur at runs of identical bases in the DNA (Ghosal and Saedler, 1978), therefore, mutations at these sites carry little phylogenetic weight.

Table 2-2. Control region sequences from mitochondrial DNA of North American wapiti, Asian sika deer, and European red deer. Nucleotide substitutions are given and gaps are indicated by -.

```
                                                                                                  60
wapiti          TAATATACTG GTCTTGTAAA CCAGAAAAGG AGAGCAACCA ACCTCCCTAA GACTCAAGGA
sika deer       .......... .......... .......... .......... .......... ..........
red deer        .......... .......... .......... ........T. .......... ..........
                                                                                                 120
wapiti          AGAAGCCATA GCCCCACTAT CAACACCCAA AGCTGAAGTT CTATTTAAAC TATTCCCTGA
sika deer       .......... .......... .......... .......... .......... ..........
red deer        T......... .......... .......... .......... .......... ..........
                                                                                                 180
wapiti          CGCTTATTAA TATAGTTCCA TAAAAATCAA GAACTTTATC AGTATTAAAT TTCCAAAAAA
sika deer       .......... .......... .......... .......... .......... ..........
red deer        .......... .......... .......... .......... .......... .........G
                                                                                                 240
wapiti          -TTTAATATT TTAATACAGC TTTCTACTCA ACATCCAATT TACATTTTAT GTCCTACTAA
sika deer       T......... .......... .......... ...C...... ......C... .C....T...
red deer        T......... .C........ ....C..... ...C...T.. .........C A...-..C..
                                                                                                 300
wapiti          TTACACAGCA AAACACGTGA TATAACCTTA TGCGCTCGTA GTACATAAAA TCAATGTGCT
sika deer       CC.....A.. ..G....... .......... ..T...T... .......... .T....CATC
red deer        .......A.. ...T.T..A. ..A....... ......TA.. .......G.. .T.....A..
                                                                                                 360
wapiti          AGGACATGC- ATGTATAACA GTACATGAGT TAGCG-TATA GGACATATTA TGTATAATAG
sika deer       .A.....A.T .....CGGT. ........AA CC.GTA.... .......C.. ..........
red deer        .......A.T .......... ......---- ---------- ---------- ----------
```

Table 2-2 con't. Control region sequences from mitochondrial DNA of North American wapiti, Asian sika deer, and European red deer. Nucleotide substitutions are given and gaps are indicated by -.

```
                                                                                                  420
wapiti          TACATAAATT AATGTATTAA GACATATTAT GTATAATAGT ACATTATATT ATATGCCCCA
sika deer       .......... .........G A......... .......... .......... ..........
red deer        ---------- ---------- ---------- ---------- ----...... ..........
                                                                                                  480
wapiti          TGCTTATAAG CATGTACTTC TCACTATCTG AAGTACATAG TACATAATGT TGTTCATCGT
sika deer       .......... ......T..T CT.T.....A T......... .....G.... CA..T.....
red deer        ...A...... .........T CT.T...T.A T......... .....G.... .........
                                                                                                  540
wapiti          ACATAGTACA TTAAGTCAAA TCAGTCCTTG TCAACATGC GTATCCCGTCC CCTAGATCAC
sika deer       ......C... .......... .......... .......... .......... ..........
red deer        ......CG.. .......... .......... .......... .......... .........
                                                                                                  600
wapiti          GAGCTTAATT ACCATGCCGC GTGAAACCAG CAACCCGCTG GGCAGGGATC CCTCTTCTCG
sika deer       ......G... .......... .......... .........A .......... ..........
red deer        ......G... .......... .......... .......... .......... ..........
                                                                                                  660
wapiti          CTCCGGGCCC ATGAACCGTG GGGGTAGCTA TTTAATGAAT TTTATCAGAC ATCTGGTTCT
sika deer       .......... .......... ......A... .........C .......... ..........
red deer        .......... ......T... .......... .........C .......... ..........
                                                                                                  720
wapiti          TTTTTCAGGG CCATCTCATC TAAAATCGCC CACTCCTTGT AAC-ATAAGA CATCTCGATG
sika deer       .......... .........C. .......... .........C ..TT.C.... ..........
red deer        .......... .........C. .......... .........C ..T-...... ..........
                                                                                                  780
wapiti          GACTAATGAC TAATCAGCCC ATGCTCACAC ATAACTGTGG TGTCATACAT TTGGTATTTT
sika deer       .......... .......... .......... .......... .......... ..........
red deer        .......... .......... .......... .......... .......... ..........
                                                                                                  840
wapiti          TAATTTTTGG GGGGATGCTT GGACTCAGCA ATGGCCGTCT GA-GGCCCCG TCCCGGAGCA
sika deer       .......... .......... .......... .......... ..-....... ..........
red deer        .......... .......... .......... ...A...... .GC..T.... ..........
                                                                                                  900
wapiti          TGAATTGTAG CTGGACTTAA CTGCATCTTG AGCATCCCCA TAATGGTAGG CGCAGGGCAT
sika deer       .......... .......... .......... .......... .......... ATG.......
red deer        .......... .......... .......... .......... .......... AT-.......
                                                                                                  960
wapiti          TACAGTCAAT GGTCACAGGA CATAGTTATT ATTTCATGAG TCAACCCTAA GATCTATTTT
sika deer       .G........ .......... .......... .......... .......... ..........
red deer        GG........ .......... ....A.C... .......... .......... ..........
                                                                                                 1020
wapiti          CCCCCCCCTT CTTATTTTTT CCCCCTTATA TAGTTATCAC CATTTTTAAC ACACTTTCCC
sika deer       .......... .......... .......... ......C..T .......... .......T..
red deer        .........G ..A....... .......... .......... .......... .......T..
                                                                                                 1080
wapiti          CTAGATATAA TTTTAAATTT ATCACATTTC CAATACTCAA AATAGCACTC CAGAGGGAGG
sika deer       ........T. .......... .......... .......... .T........ ..........
red deer        ........T. .......... .......... .......... .TC....... ...G...T..
                                                                                                 1140
wapiti          TAAGTATATA AACGCCAATT TTTCCCTAAT TATGCATAGT TAATGTAGCT TAAACAGCAA
sika deer       .......... .......... .......... .......... .......... ..........
red deer        ...C...... .......... .......... .....T.C.. ..G....... ....TG....
                                                                                                 1200
wapiti          AGCAAGGCAC TGAAAATGCC TAGATGAGTA TATTAACTCC ATAAAACACA TAGGTTTGGT
sika deer       .......... .......... .......... .......... .......... ..........
red deer        .......... .......... .......... .......... .......... ..........

wapiti          CCCAGCCTTC CGACCC
sika deer       .......... ......
red deer        .......... ......
```

Table 2-3. Nucleotide substitutions in the control region of mitochondrial DNA among North American wapiti subspecies. Nucleotide substitutions that vary from the wapiti consensus sequence are given, where deletions are indicated with --.

	Position of Nucleotide Substitution																			
Wapiti	113	181	269	315	440	442	444	448	450	476	487	488	493	541	627	679	681	694	703	709
Consensus	T	--	T	A	C	C	C	C	G	A	T	A	A	G	G	T	T	T	C	G
Riding Mt.1	.	--	.	G	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Riding Mt.2	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Riding Mt.3/4	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Riding Mt.5	A	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Riding Mt.7	.	A	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
French 5A	.	--	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	A
French B6	.	--	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	.
BurwashT1/5(55)	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Elk Island 20	.	--	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Elk Island 63	.	--	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Elk Island 72	.	--	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Banff 14	.	--	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	C	.	.
Banff 23	.	--	.	.	.	.	T	T	A	.	C	G	.	.	.	.	.	.	.	.
Banff 37	.	--	.	.	.	.	T	T	A	.	C	G	.	.	.	.	.	.	.	.
KNP/Yellowstone 2	.	--	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Jasper 91	.	--	.	.	.	.	.	.	A	.	C	.	.	.	.	.	.	.	.	.
Jasper 92	.	--	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.
Yellowstone 1	.	--	.	.	T	.	.	.	A	.	.	.	.	.	.	.	.	C	.	.
Roosevelt 23	.	--	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.
Roosevelt 25	.	--	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.
Roosevelt 29	.	--	.	.	.	.	.	.	A	.	.	.	G	.	.	.	.	.	.	.
Roosevelt 32	.	--	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.
Roosevelt 33	.	--	.	.	.	.	.	.	A	.	.	.	G	A	.	.	.	.	.	.
Tule 457	.	--	C	.	.	.	.	.	.	.	.	.	.	.	A	.	A	.	T	.
Tule 659	.	--	C	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	T	.
Sika deer 226	.	T	.	.	T	T	T	.	A	.	C	.	.	.	A	C	.	.	T	.
Sika deer 215	.	T	.	.	T	T	T	.	A	.	C	.	.	.	A	C	.	.	T	.
Red deer 765	.	T	.	.	T	T	T	.	A	.	C	G	.	.	.	C	.	C	T	.
Red deer 923	.	T	.	.	T	T	T	.	A	.	.	G	.	.	.	C	.	.	T	.

Table 2-3 con't. Nucleotide substitutions in the control region of mitochondrial DNA among North American wapiti subspecies. Nucleotide substitutions that vary from the wapiti consensus sequence are given, where deletions are indicated with --.

	Position of Nucleotide Substitution																			
Wapiti	717	737	798	808	838	852	867	935	942	951	960	968	981	986	988	1024	1053	1116	1137	1153
consensus	G	G	C	G	G	T	C	C	C	G	T	C	--	C	T	G	A	A	G	A
Riding Mt.1	.	.	.	.	.	.	.	.	.	.	.	--	--	.	.	.	.	.	.	.
Riding Mt.2	.	C	.	.	.	.	.	.	.	.	--	--	--	.	.	.	.	.	.	.
Riding Mt.3/4	.	.	.	.	.	.	.	.	.	.	.	--	T	.	.	.	.	.	.	.
Riding Mt.5	.	C	.	.	.	.	.	.	.	.	--	--	--	.	.	.	.	.	.	.
Riding Mt.7	.	.	.	.	A	.	.	.	G	.	.	--	T	.	.	.	.	.	.	.
French 5A	.	.	.	.	.	.	.	.	.	.	.	--	T	.	.	.	.	.	.	.
French 6	.	.	.	.	.	.	.	.	.	.	.	--	T	.	.	.	.	.	.	.
BurwashT1/5(55)	.	.	.	.	.	.	.	.	.	.	--	--	--	.	.	.	.	.	.	.
Elk Island 20	C	.	A	T	.	.	T	.	.	.	.	--	T	.	.	.	.	.	.	.
Elk Island 63	.	.	.	.	.	.	.	.	.	.	.	--	T	.	.	.	.	.	.	.
Elk Island 72	C	.	.	T	.	.	T	.	.	.	.	--	T	.	.	.	.	.	.	.
Banff 14	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Banff 23	.	.	.	.	.	.	.	.	.	A	.	.	--	.	.	.	G	G	A	.
Banff 37	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
KNP/Yellowstone2	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Jasper 91	.	.	.	.	.	.	.	T	.	.	.	.	--	.	.	.	.	.	.	.
Jasper 92	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Yellowstone 1	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Roosevelt 23	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	T
Roosevelt 25	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	T
Roosevelt 29	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	A	.	.	.	T
Roosevelt 32	.	.	.	.	.	.	.	.	.	.	.	.	--	T	C	.	.	.	.	.
Roosevelt 33	.	.	.	.	.	.	.	.	.	.	.	.	--	.	C	.	.	.	.	T
Tule 457	.	.	.	.	.	G	.	.	.	.	--	.	--	.	.	.	.	.	.	.
Tule 659	.	.	.	.	.	.	.	.	.	.	--	.	--	.	.	.	.	.	.	.
Sika deer 226	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Sika deer 215	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Red deer 765	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.
Red deer 923	.	.	.	.	.	.	.	.	.	.	.	.	--	.	.	.	.	.	.	.

The sequence of Yellowstone National Park wapiti (2) matched that of the KNP wapiti; Riding Mountain National Park sample 3 matched Riding Mountain National Park sample (Riding Mt. 4); and Burwash sample T1 matched Burwash sample T5(55). Because mtDNA is passed maternally, animals sharing female founders will have the same mtDNA sequences. The Riding Mountain National Park wapiti 3 and 4 were from the same herd, and similarly, the Ontario wapiti T1 and T5(55) were from the same herd. The KNP wapiti most likely descended from a Yellowstone animal relocated to Banff in the 1920's.

The observed number of nucleotides that varied between sequences ranged from 2 - 14 nucleotides among Rocky Mountain and Manitoban animals, from 3 - 13 nucleotides among Rocky Mountain and Roosevelt, and from 4 - 15 nucleotides among Rocky Mountain and Tule wapiti, with an average of 0.56 % (6.53/1165) nucleotide substitutions between wapiti. As estimated from sequence divergence, Manitoban and Rocky Mountain wapiti had the closest genetic distance where $\pi = 0.00767$, followed by Rocky Mountain and Tule where $\pi = 0.00826$. The greatest distances were found between Tule wapiti, where $\pi = 0.01256$ to Manitoban and $\pi = 0.01288$ to Roosevelt wapiti. A comparison among the number of nucleotide differences of all subspecies places Rocky Mountain wapiti central to all other subspecies (Table 2-4).

Table 2-4. Divergence of mtDNA D-loop sequences from wapiti subspecies as calculated from the number of nucleotide differences between individuals from each type. Values in bold represent the sequence variation within the subspecies (d_X), values above the diagonal represent uncorrected sequence variation within the species (d_{XY}), and values below the diagonal represent the sequence divergence between subspecies when corrected for intra-specific variation (π).

	Wapiti Subspecies			
	Manitoban	Rocky Mountain	Roosevelt	Tule
Manitoban	**0.00326**	0.01110	0.01344	0.01505
Rocky Mountain	0.00767	**0.00343**	0.01047	0.01084
Roosevelt	0.01044	0.01257	**0.00275**	0.01511
Tule	0.01257	0.00826	0.01288	**0.00172**

Comparisons of nucleotide substitutions (Table 2-3) in Tule wapiti revealed that site 269 was unique to Tule wapiti, site 627 was shared with sika deer, and site 703 was shared with both red deer and sika deer. Comparisons of nucleotide substitutions in Roosevelt wapiti showed site 493 was unique to all members of this group, site 1154 was unique to most of the Roosevelt, and site 450 was shared among several Roosevelt and Rocky Mountain wapiti. There were no informative nucleotide sites shared among all members of either the Rocky Mountain or Manitoban wapiti, although the insertion at site 981 and deletion at site 960 were exclusive to several Manitoban wapiti. Nucleotide

substitutions at sites 487 and 968 were found among both Rocky Mountain and Manitoban wapiti. The nucleotide change at site 488 created a unique recognition site for endonuclease *Cfo I* present among several Rocky Mountain wapiti. The nucleotide substitution at site 493 identified the unique recognition site for *Hinf I* found in Roosevelt wapiti.

Trees were constructed using equally and unequally weighted characters, and both including and excluding gaps (Fig 2-3). Roosevelt and Tule wapiti were found in monophyletic clades regardless of constraints or weights. If gaps were not considered informative and transversions were not given extra weight, then both Manitoban and Rocky Mountain animals were found to be paraphyletic. By including gaps as new character states but no extra weighting on transversions, the heuristic search placed Rocky Mountain animals into clades that (*i*) branch before all other animals (Yellowstone 1, Banff 23, Banff 37, and Jasper 91), (*ii*) include Manitoban animals (Banff 14 and Jasper 92), and (*iii*) formed a sister-clade to Tule and Roosevelt animals. This would make the Rocky Mountain group polyphyletic while the Manitoban group would remain paraphyletic. Weighting transversions twice as heavily as transitions, and including gaps as characters, also resulted in the paraphyly of Manitoban and polyphyly of Rocky Mountain types (see Figure 2-3a). Weighting transversions to transitions more strongly (5:1 or 10:1) and counting gaps caused some interesting changes not illustrated in Figure 2-3: Elk Island animals 20 and 72 grouped closely with the Yellowstone 1 animal in a clade that also included Riding Mountain National Park individuals; Yellowstone 2 and Kootenay National Park animals move to a clade containing both Tule and Roosevelt animals. Banff 14 and Jasper 92 National Park animals again grouped with the remaining Riding Mountain and Burwash River animals; and Banff 23, Banff 37, and Jasper 91 National Park animals branched early in the tree. Both Manitoban and Rocky Mountain groups became polyphyletic with increasing weights on transversions.

The consensus of 26 equally parsimonious trees using transversions weighted twice that of transitions and gaps treated as new character states shown in Figure 2-3a illustrates the relationships observed among wapiti common to most trees of weighted and non-weighted characters. The consistency index was 0.928 and branch lengths were equal to 262 steps.

It is important to note that the Yellowstone 2/Kootenay National park animal can be moved to the clade containing the Manitoban wapiti without additional steps by changing the order characters 968 and 487 appear in the tree. Branch lengths varied between 230 - 562 for bootstraps on unweighted trees that saved only one tree per replication. Bootstraps for 100 replications were performed using a ratio of 2:1 for

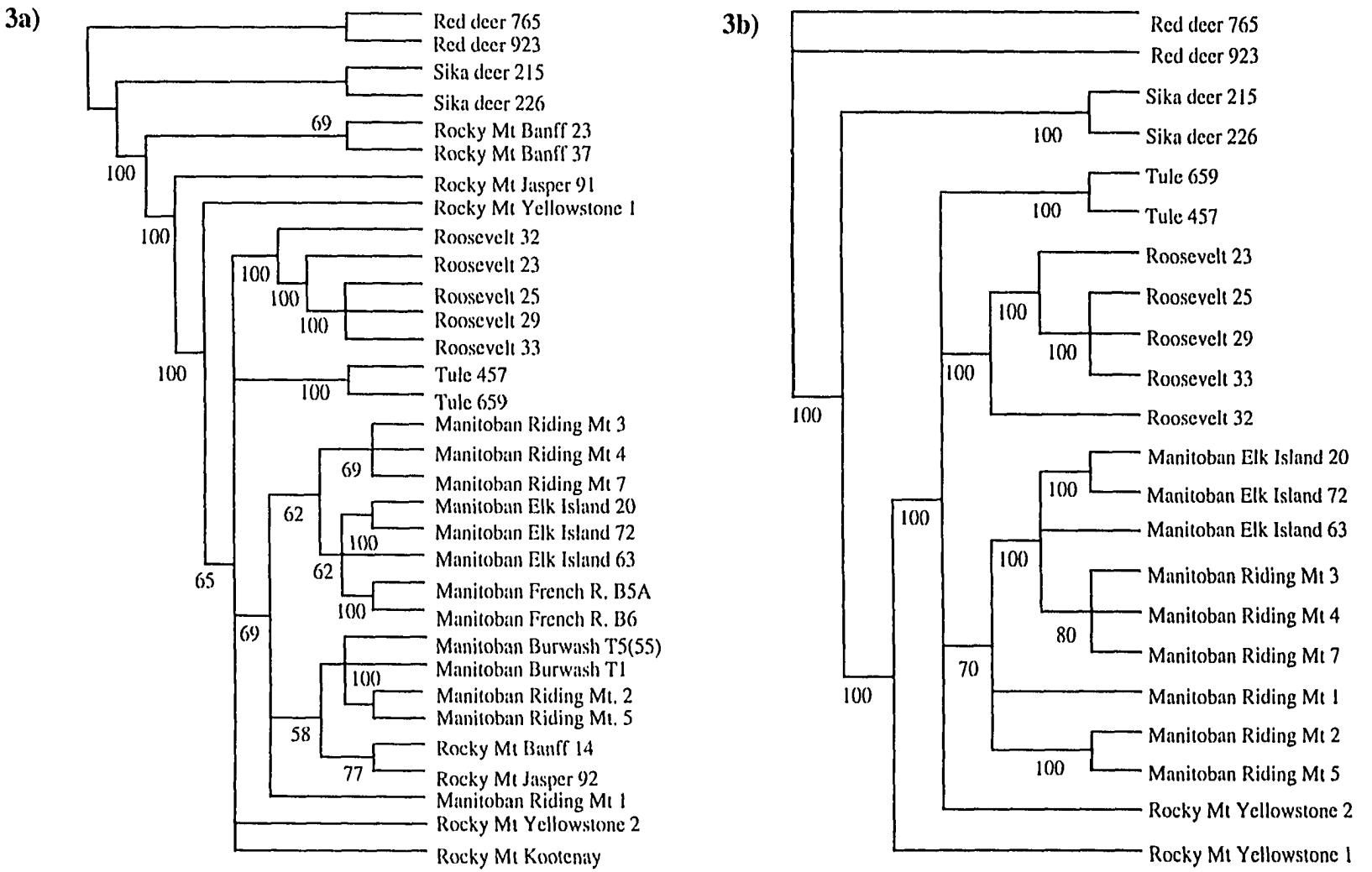


Figure 2-3. a) The inferred phylogenetic relationship among North American wapiti based on the D-loop region of mitochondrial DNA. The 50 % majority rule consensus of 26 most parsimonious trees, using a 2:1 weighting of transversions to transitions, requires 262 steps and has a CI = 0.928. b) The phylogenetic relationship among pure populations of North American wapiti as based on the D-loop region of mitochondrial DNA. The majority rule consensus of 10 most parsimonious trees, using a 2:1 weighting of transversions to transitions, requires 225 steps and has a CI = 0.951.

transversions to transitions found in wapiti where branch lengths varied from 230 - 562 steps. The bootstrap (not shown) used 280 steps and had a CI = 0.821. Roosevelt wapiti were grouped together with 56 % frequency in the weighted bootstrap consensus tree. Rocky Mountain wapiti from Jasper, Kootenay, Yellowstone, and Banff 14 and the Manitoban sample Riding Mountain 1 also did not sort into any one clade. The number of homoplasies and absence of unique informative characters does not lead to a consistent division of Rocky Mountain or Manitoban wapiti into subspecies.

Analysis of populations that have had no introductions, including Elk Island, Riding Mountain, and Yellowstone National parks, was also performed using the same restraints as are noted above. Rocky Mountain wapiti were paraphyletic and Manitoban, Tule, and Roosevelt wapiti were monophyletic (Figure 2-3b): this assumes that Elk Island animals are of the Manitoban type.

Restriction digests of the ten Olympic Peninsula Roosevelt samples using the enzyme *Hinf I* f revealed six individuals with fragment sizes of approximately 450, 340, 300, and 135 base pairs, and four individuals with fragment sizes of 750, 340, and 135 base pairs. The first restriction fragment length pattern was formerly found among only Roosevelt individuals while the second was common to both Rocky Mountain and Manitoban forms. *Hinf I* sites can be found at sequence sites 493, 809, 906, and 1148.

Discussion

Historically, North American wapiti populations were assigned to their respective subspecies largely on the basis of their geographic distribution, which has made the taxonomic classification particularly suspect for the Rocky Mountain, Manitoban, eastern, and Merriam wapiti. The phylogenetic relationship of wapiti in this study is discussed with regards to the invasion and distribution of wapiti into North America and the large number of re-introductions of animals into both historical and nonhistorical ranges.

Wapiti originated in Asia and entered North America by crossing the Bering land bridge (Guthrie 1966). The land bridge between these two continents was thought to have disappeared 10,000 - 15,000 years ago when the sea level rose (Pielou 1991). The post Wisconsin stage (10,000 - present) was marked by gradual climate and habitat changes that may have led to the extinction of the Alaskan population, the division of the large central population into a montane/boreal, prairie, and deciduous forest ecotypes, and the further isolation of the Californian and west coast populations by the Cascade and Rocky Mountains (Guthrie 1966). Prior to the arrival of Europeans, Seton estimated 10,000,000

wapiti in North America, with numbers dwindling to less than 100,000 by 1907 (Bryant and Maser 1982). Both numbers were likely overestimates, but they illustrate that wapiti were once widely distributed across North America, with the exception of the Tule and Roosevelt wapiti residing along the west coast. According to the phylogenetic tree, all wapiti subspecies appear to descend from one common ancestor, which clearly suggests a close relationship among the North American animals.

As the wapiti population expanded and herds dispersed into new habitats, eventually a few founders would have moved into the remote coastal regions that gave rise to the Tule and Roosevelt populations. Murie (1951) suggested the Rocky Mountain wapiti possibly gave rise to the Tule and Roosevelt wapiti, although Bailey (1936:78) found no fossil records to indicate that the range of Rocky Mountain wapiti was ever connected with the Roosevelt animals. Movement across the mountain ranges was not impossible, but likely not extensive. Both Roosevelt and Tule populations are monophyletic which suggests they each were derived from single lineages. Populations isolated for long periods of time generally accumulate nucleotide differences not found in other populations. These differences translate into greater genetic distances between populations. The greatest number of nucleotide differences were found in comparisons with Tule and Roosevelt animals. Tule and Roosevelt wapiti (Vancouver Island) have maintained their monophyletic status as a result of isolation brought about by habitat changes, reduction in their populations caused by human intervention, and the fortuitous lack of relocations of wapiti into or out of these populations.

The Roosevelt population from the Olympic Pennisula, WA is comprised of a mixture of individuals with haplotypes unique to Roosevelt and haplotypes common to Rocky Mountain and Manitoban forms. Presently, Washington state is home to large populations of both Roosevelt and Rocky Mountain animals (Bryant and Maser 1982), and movement between the two populations may account for the presence of Rocky Mountain/Manitoban haplotypes in the Olympic Penninsula National Park. The introduction of Yellowstone wapiti into the Wenatchee Mountains between 1913 - 1933 would have placed the Rocky Mountain wapiti within a reasonable travelling distance to the Olympic population. Combining restriction data from Polziehn (1993), where eight Olympic Peninsula National Park animals also had the unique *Hinf I* restriction site, the frequency of Rocky Mountain/Manitoban haplotypes in the Olympic population is 22 %.

A panmictic or a clinal distribution was suggested for the Eastern, Manitoban, and Rocky Mountain wapiti (Bryant and Maser 1982; Schonewald 1994). With a few exceptions, Schonewald (1994) found a decrease in cranial size from north to south and from western Europe to North America. Blyth and Hudson (1987) suggested the

parkland area of central Alberta serves as a transition zone between boreal and prairie habitats, and that Rocky Mountain and Manitoban wapiti in Alberta may share genetic affinities because of overlapping ranges. A similar relationship between the historical ranges of the Manitoban and Eastern subspecies can be suggested.

Elk Island National Park wapiti were assumed to be Manitoban types because their mtDNA grouped with other Manitoban wapiti, and the Manitoban range was thought to extend into this region. However, animals used to describe the Rocky Mountain type by Bailey (1935) included wapiti from Fort Saskatchewan, which is approximately 20 km west of Elk Island National Park. Perhaps the Manitoban wapiti ranged further west than previously believed, and animals from Fort Saskatchewan should not have been included in the Rocky Mountain group. However, the Elk Island National Park animals most likely represent animals in the transition zone, which have morphological and genetic similarities to both types. This population is likely the greatest proof that Rocky Mountain and Manitoban subspecies are the least differentiated subspecies of wapiti.

Yellowstone, Elk Island, and Riding Mountain National Parks have not introduced animals from outside sources into their resident populations. Using only these three populations, one would conclude a monophyletic origin for Manitoban wapiti, assuming both Elk Island and Riding Mountain populations are Manitoban types. The Rocky Mountain population would be paraphyletic as one lineage branches early in the tree and another branch shares a node with all other forms. This study provides support for the Manitoban subspecies status of wapiti in the Elk Island and Riding Mountain National Parks. The sample size from Yellowstone is however too small to draw any strong conclusions regarding the relationship of Rocky Mountain to Manitoban wapiti.

Using the complete data set, which assumes wapiti in the Rocky Mountains are most likely of this type, results in a phylogeny which places a few Rocky Mountain and Manitoban types in the same clade. This suggests the separation between these two groups is only in the early stages of development. The longer populations are isolated, the more likely shared lineages are lost, and a transition from polyphyly to paraphyly to monophyly will occur. The Rocky Mountain animals (Banff 14 and Jasper 92) found within the clade containing Manitoban wapiti are more likely descendants from Yellowstone National Park as all Elk Island animals were transplanted outside these parks. The lowest genetic distances were found between these two subspecies which also suggests that any separation between these two subspecies is recent.

In the comparison of sequences from the mtDNA D-loop region, Burwash and French River wapiti presently living in the range of the extinct Eastern wapiti, were placed in the same clades as Manitoban or Manitoban/Rocky Mountain group. The absence of

unique differences among the D-loop sequences suggests the Burwash and French populations are likely not the same as those formerly belonging to the Eastern wapiti. The founders of these recent populations originate from the Wainwright herd, which contained descendants from Montana, Wyoming, and/or Ontario. If the Burwash and French animals descend from lineages that could be directly linked to either Montana or Wyoming origins, both Rocky Mountain and Manitoban types would be polyphyletic.

Outside of the park boundaries, one would expect to find even less support for a distinction between Rocky Mountain and Manitoban subspecies. The Rocky Mountain wapiti in Canada are surrounded by transplants of Elk Island National Park animals, and are most likely to show hybridization. However, isolated populations of Yellowstone National Park in the United States should represent true Rocky Mountain types. Neither Elk Island or Riding Mountain National Parks have had Rocky Mountain animals released within their borders, but both populations have the potential to hybridize with free-ranging and game ranched animals with Rocky Mountain origins.

Overall, there is a clear lack of mtDNA variation within North American wapiti, which corresponds well with previous genetic studies and the lack of morphological differences. The average 0.560 % genetic difference in the mtDNA among North American wapiti is comparable to the 0.364 % (2/549 nucleotides) observed in North American moose (*Alces alces*; Mikko and Andersson 1995) but substantially less than the 2.5 % in white-tailed deer (*Odocoileus virginianus*; Ellsworth *et al.* 1994).

This phylogenetic study has shown that there is a slight distinction between pure wapiti populations, most likely because of the limited number of founders and the absence of wapiti introductions into these populations. Both Roosevelt from Vancouver Island and Tule wapiti were monophyletic which, by definition, supports their subspecific status. In the absence of geographic barriers, hybridization likely took place at some time between neighboring Rocky Mountain and Manitoban animals, and both types are found within one clade. The lack of distinction between some Rocky Mountain and Manitoban animals would suggest that these two groups are at the early steps of subspeciation.

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Chapter 3

Microsatellite analysis of North American wapiti (Cervus elaphus) populations[1]

Abstract

Eleven populations of wapiti (*Cervus elaphus*) were analyzed for genetic diversity using 12 microsatellite loci. Samples were taken from Vancouver Island, BC; Burwash and French River herds in Ontario; Ya Ha Tinda Ranch, AB; and Banff, Elk Island, Jasper, Kootenay, Riding Mountain, Yellowstone, and Yoho National Parks. Overall, wapiti populations have on average 3 to 4 alleles per locus and an average expected heterozygosity that ranged from 26 % to 53 %. The greatest genetic distances were observed between the Vancouver population and all other populations. Using the assignment test, Roosevelt wapiti (*C. e. roosevelti* Merriam 1897) assigned only to the Vancouver Island population. The distance and assignment values suggest a divergence of the Roosevelt wapiti from other populations and support the subspecific status for the Vancouver Island population. No evidence was found for the existence of unique Eastern wapiti (*C. e. canadensis* Erxleben 1777) in the Burwash or French River herds in Ontario. The overlapping distribution of genotypes from indigenous populations from Riding Mountain, Elk Island, and Yellowstone National parks suggests wapiti were once a continuous population before settlers decimated their numbers. The lack of differentiation between these populations raises questions about the status of Manitoban (*C. e. manitobensis* Millais 1915) and Rocky Mountain (*C. e. nelsoni* Bailey 1935) subspecies.

Introduction

Pressure to manage and conserve wild populations has added to the explosion of information on the biodiversity of species, where the distribution of genetic variability is commonly used to verify species, subspecies or population division. Monitoring change in diversity also may be useful for predicting populations in peril (Luikart *et al.*

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[1] A version of this text is in press. Polziehn RO, Hamr J., Mallory FF, (2000) *Molecular Ecology*, 9.

1998) because the persistence of a population partially depends on maintaining its evolutionary significance, which requires genetic variation (Frankel & Soulé 1981). Population bottlenecks have been a major cause of reduced variation in populations, although migration or supplementation of animals can maintain genetic variability (Lacy 1987). North American wapiti *(Cervus elaphus* subspecies) were not excluded from events that reduced and fragmented populations, and local extinctions occurred soon after the arrival of European settlers (Bryant & Maser 1982). However, wapiti prospered in the protected environments of national parks, and since the early 1900's they have been relocated to areas historically known to support wapiti. Today, however, the issues of genetic variability and subspecies distinction of wapiti should be addressed before further relocations are attempted.

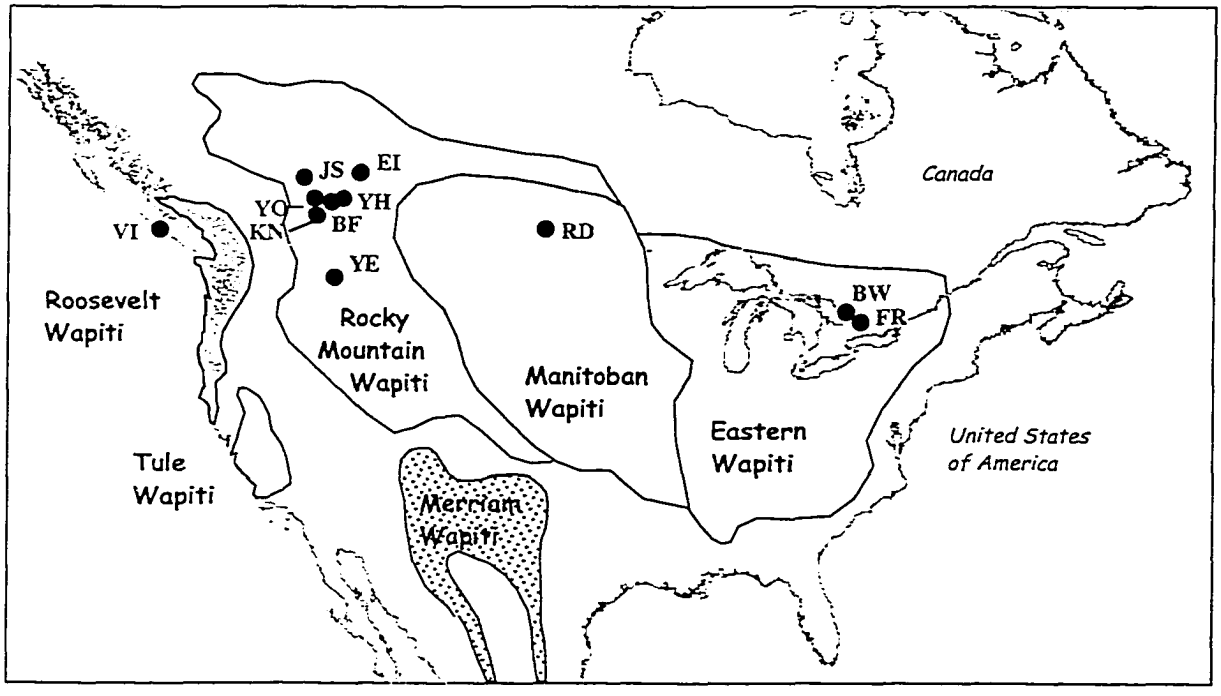

Figure 3-1. Historic distributions of wapiti subspecies in Canada and the United States, as adapted from Bryant and Maser (1982). Shown are the Roosevelt, Rocky Mountain, Manitoban, Eastern, Tule, and Merriam subspecies. The approximate sample locations include Vancouver Island (VI), Kootenay (KT), Yoho (YO), Jasper (JS), Elk Island (EI), Banff (BF), Ya Ha Tinda Ranch (YH), Yellowstone (YE), Riding Mountain (RD), French River (FR), and Burwash (BW).

The distribution of wapiti subspecies and the populations sampled in this study are shown in Figure 1. Sample localities that represent subspecies of *Manitoban (C. e. manitobensis* -Millais 1915), Rocky Mountain (*C. e. nelsoni* -Bailey 1935), and

Roosevelt (*C. e. roosevelti* -Merriam 1897) wapiti are respectively, Riding Mountain National Park, Manitoba; Yellowstone National Park, Wyoming; and Vancouver Island, British Columbia. Elk Island National Park, Alberta animals fall into the range of Rocky Mountain wapiti (Bryant & Maser 1982), however their mtDNA lineages are closer to the Manitoban type (Polziehn *et al.* 1998, Chapter 2). These four populations play an important role in studies of genetic variation from natural populations of wapiti because they have not exchanged animals and they represent four of the largest populations that survived the extirpation events of the 1700's through to the 1900's.

Several wapiti populations included in this study were founded from relocated animals. Populations that were established from relocated Yellowstone animals were Jasper and Banff National Parks, Alberta (Lloyd 1927, Stelfox 1993). Wapiti located in Kootenay and Yoho National Parks are most likely offspring of Elk Island animals released in the Kootenay valley in 1946 or animals transplanted from Banff in 1970 and 1971 (Stelfox 1993) because records indicate few indigenous wapiti were found in interior British Columbia in the early 1900's (Spalding 1992). Likewise few indigenous herds of Eastern wapiti were observed in Ontario at the turn of the century (Bryant & Maser 1982). The relocation of animals from Buffalo Park, Alberta to Ontario and subsequent introduction into the wild, albeit not intended, has led to the establishment of populations in Burwash and French River, Ontario. These latter two populations were included in this study on the presumption they may be supplemented with descendants of the extinct Eastern wapiti once found in Ontario.

The genetic diversity of wapiti populations has been measured using variation in proteins (Cameron & Vyse, 1978; Dratch 1986; Glen & Smith, 1993) and mitochondrial DNA (mtDNA) restriction fragments (Cronin, 1993; Polziehn *et al.* 1998; Chapter 2). Both techniques detected little to no variation and only hinted at subspecies differentiation. By sequencing the control region of mtDNA, unique lineages for Tule (*C.e.nannodes*) and Roosevelt wapiti were observed and marginally more variation was detected (Polziehn *et al.* 1998). Microsatellite analysis has been successfully applied to study *C. elaphus* populations (Marshall *et al.* 1998) and populations thought to be genetically depauperate (Hughes & Queller, 1993; Paetkau *et al.* 1995). In this study, twelve microsatellite loci were used to assess the genetic diversity of indigenous and relocated wapiti populations and to determine if the distribution of diversity will uphold the current status of wapiti subspecies.

On a finer scale, microsatellites can be used to assess relationships among demes within a population (Wilson & Strobeck 1999). In contrast to an island population that has a well-defined range, the distribution of a herd or deme is difficult to

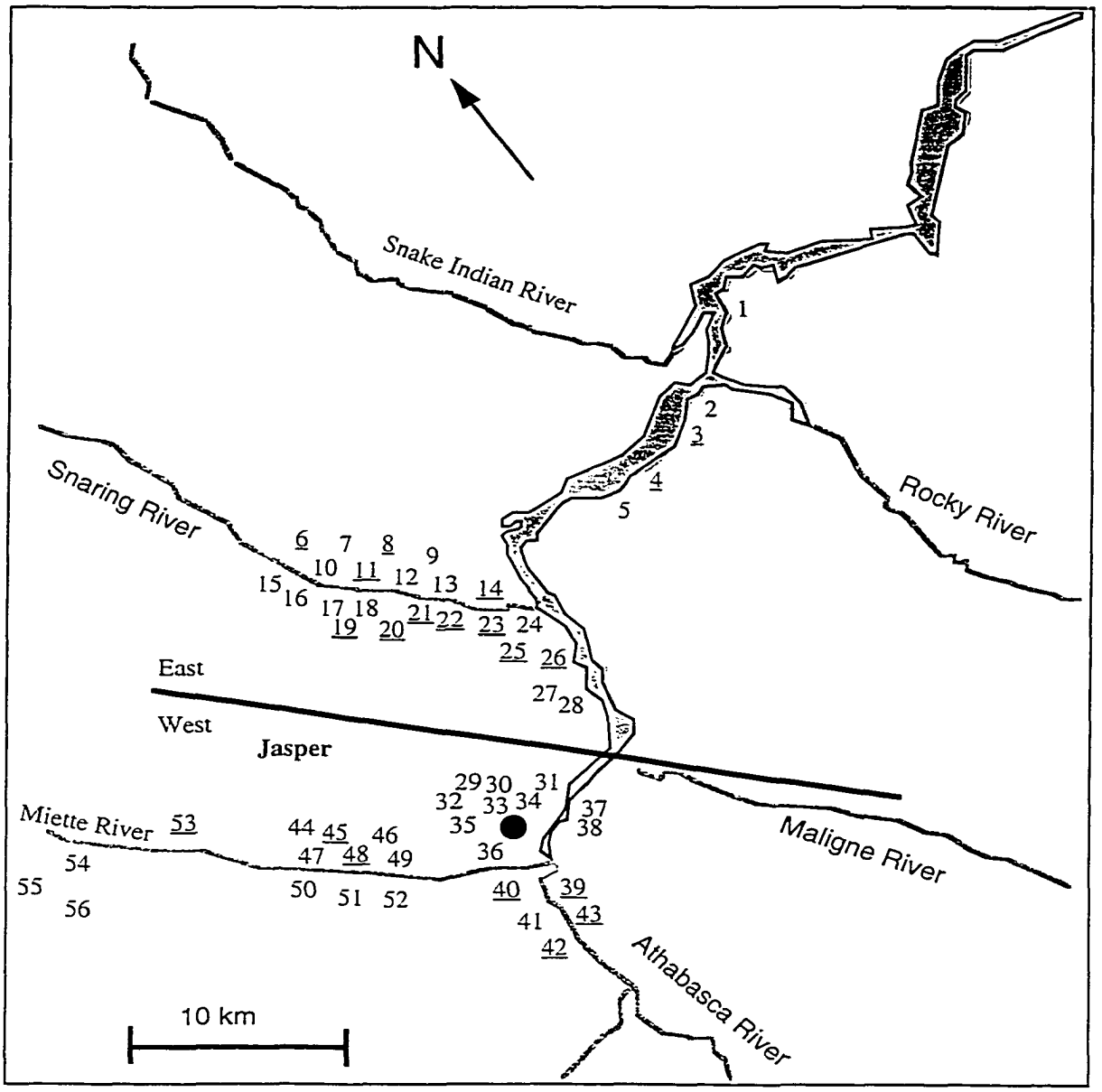

Figure 3-2. Relative sampling locations of wapiti from Jasper National Park. Individuals that were correctly assigned within the park are underlined.

characterize. The term 'herd' is used in this study to describe animals found in the same location and does not necessarily imply any familial relationship, although common belief is that *Cervus elaphus* herds are comprised of closely related females (Clutton-Brock *et al.* 1982). If the amount of relatedness in a herd decreases as density increases or as animals age (Albon *et al.* 1992), large established populations should show little genetic structure. DNA profiles were compared among wapiti distributed along the Miette and Athabasca River corridor of Jasper National Park, Alberta and the

relatedness of animals was used to determine the presence of subdivision within the large Jasper population.

Materials and Methods

Sample Collection

Muscle tissue was collected from animals killed by motor vehicles or trains by park wardens at Banff, Jasper, Kootenay, Riding Mountain, and Yoho National Parks. Sample sizes were 28, 56, 27, 36, and 15, respectively. Blood samples were obtained from 20 wapiti from Elk Island National Park as they were processed for relocation outside of the park. Joe Hamr and Frank Mallory collected three blood samples from captured animals and 11 muscle tissue samples from animals killed by trains near Burwash, Ontario and five blood samples from captured animals and five tissue samples from drowning, hunter, or wolf kill from French River, Ontario. The British Columbia Fish and Wildlife Service collected thirty muscle tissue samples from animals killed by motor vehicles on Vancouver Island. The Wyoming Fish and Wildlife and United States Parks Service collected twenty-two muscle tissue samples from hunter kills from the Teton-Yellowstone National Park area. Alberta Fish and Wildlife supplied 30 ear plug samples collected from the Ya Ha Tinda Ranch located near Banff National Park. DNA was isolated from all samples using the Qiagen QIAmp tissue isolation kit (Chatsworth, CA).

Amplification of Microsatellite Loci

PCR was used to amplify 12 microsatellite loci with AC repeats. Primer pairs BL42, BM1009, BM203, BM4207, BM4208, BM888, BM5004, and BM4513 were characterized by Bishop *et al.* (1994); primer pairs ETH152 and FCB193 were described respectively by Steffen *et al.* (1993) and Buchanan and Crawford (1993); and primer pairs RT13 and RT1 were described by Wilson *et al.* (1997). The first seven loci described by Bishop *et al.* (1994) and two loci by Steffen *et al.* (1993) were chosen for paternity testing in North America and were selected from 14 loci found to be polymorphic when testing 50 bovine and ovine primer sets in wapiti (Talbot *et al.* 1996). Talbot *et al.* (1996) observed an average of five alleles per polymorphic locus, with 8 alleles being the greatest number of alleles at a locus. Locus BM4513 was proven to be one of the most variable of 174 bovine loci tested in red deer (*C. elaphus*; Slate *et al.* 1998). Loci RT 1 and RT 13 were also selected on the basis of their variability in North American wapiti (Wilson *et al.* 1997).

Primers were synthesized on an ABI 391 DNA Synthesizer, and one primer of each pair was labeled with a fluorescent dye. The dyes allowed the PCR products to be detected and sized on an ABI 377 DNA sequencer. A size standard labeled with TAMRA was used to estimate the sizes of the microsatellites and included amplified products of the following lengths: 80, 130, 180, 230, 280, 330, and 380 bp. Locus RT13 was singly amplified, and loci RT1 and BM451.3 were co-amplified using the PCR cocktails described by Wilson *et al.* (1997). These three loci were multiplexed for each sample in a single gel lane. The PCR cocktails for the co-amplification of loci of BL42, BM1009, and BM203; the co-amplification of loci BM4207, BM4201, BM888, and BM5004; and the co-amplification of ETH152, and FCB193 are described by Talbot *et al.* (1996). These nine loci from each sample were multiplexed in another single gel lane, therefore two lanes were required to resolve all 12 markers for each individual.

Amplification of DNA on a Perkin Elmer Cetus 9600 was successful for all loci using the schedule outlined below. Samples were heated to 94 °C for 1 min., followed by three cycles of 94 °C for 30 s, 54 °C for 20 s, and 72 °C for 1 s, followed by 33 cycles of 94 °C for 15 s, 58 °C for 20 s and 72 °C for 1 s. The final synthesis step was extended to 30 min. at 72 °C because it minimized the appearance of stutter bands (Smith *et al.* 1995). Dilutions necessary for either multiplex reaction described above were as follows: 2 μl of PCR mix containing Hex labeled products and/or 1 μl of each PCR mix containing TET-FAM labeled products brought to a total volume of 6 μl with double distilled water. After diluting the samples 0.8 μl of each sample mixture was added to 1.8 μl of buffer containing 5:1 formamide and size standard (described above). Samples were heated at 96 °C for 2 min., placed on ice, and loaded on a 4.5 % acrylamide gel. Data collection, analysis, and sizing was performed using ABI Prism™ Genescan™ and Genotyper™ software.

Statistical Methods

Genetic diversity for populations can be estimated from the mean number of alleles, average heterozygosity, and probability of identity (probability that two individuals drawn at random from a given population have identical genotypes). The expected and unbiased heterozygosity for a locus was estimated from the allele frequencies observed within a population locus and corrected for small population size (Nei & Roychoudhury 1974). The mean number of alleles and the average heterozygosity were calculated as the averages of these respective numbers over all loci

for each population (Nei & Roychoudhury 1974). The unbiased probability of identity was used to estimate the probability of observing an individual's genotype in the population (Paetkau *et al.* 1998). To detect deviations from the Hardy-Weinberg equilibrium for loci with three or more alleles, the Monte-Carlo approximation of the Fisher's exact test was employed (Guo & Thompson 1992). This test assumes random mating and linkage equilibrium within each population. Loci with two alleles were tested for departure from Hardy-Weinberg using the X^2 goodness- of- fit test. An excess of homozygotes at each locus can indicate the presence of null alleles, inbreeding, or population division.

Nei's standard distances (Ds; Nei, 1972) and Nei's minimum distances (Dm; Takezaki & Nei 1996) were estimated from the observed allele frequencies where absent alleles were given a frequency of 0.01 to avoid zero values. PHYLIP 3.57c (Felsenstein 1995) was used to construct Nei's distance trees using the Fitch-Margoliash (FM; Fitch & Margoliash 1967) and Neighbor Joining method (NJ; Saitou and Nei 1987). Both FM and NJ methods are additive and allow for unequal rates of mutation (Felsenstein 1995).

The assignment test (Paetkau *et al.* 1995) uses the allele distribution to calculate the probability that an individual's genotype would be found in any of the eleven populations. The significance of the assignment test results can be determined using randomization replicates. Each replicate generated by the randomization option used allele distributions observed within the population to generate an equal number of new genotypes. One thousand replicates of the randomization test were performed and the numbers of replicates were noted that have an equal or greater number of misassigned individuals as observed from the assignment test. Misassignments that occurred with a frequency Rdf $\leq$ 0.05 were considered significant, and were used to infer the presence of migrants. A population that has zero misassignments would be expected to have 1000 out of 1000 (Rdf = 1.0) replicates that have an equal (0) or greater number of misassigned individuals. However, a population that has only 15 out of 1000 replicates (Rdf = 0.015) that have an equal or greater number of incorrectly assigned individuals suggests random occurrence of some genotypes in the population is rare and likely arose from migrants into the population. Calculations for genetic distances, allele frequencies, and the assignment test can be found at http://www.biology.ualberta. ca /jbrzusto

Samples from Jasper National Park were collected along the Athabasca and Miette Rivers, starting near the east gate and extending 80 km to the west gate (Figure

3-2). Shared allele and relatedness (R) distances were calculated between individuals and used to construct NJ trees. Genetic relatedness and geographic location were correlated to test the fidelity of an animal to an area. Pairs identified as having high relatedness scores using Relatedness 5.04 (Queller & Goodnight 1989) were screened for possible parent/offspring pairs (share at least one allele per locus), however, without parentage information, individuals which were identified as parent/offspring pairs may also represent sibling pairs. In winter about 1,200 wapiti are found in the park with about 70 % of the population concentrated in the Athabasca valley (Bradford 1995). Two areas along the Athabasca valley where animals congregate are near the town of Jasper and approximately 20 km east near Snaring River.

Results

Measures of Genetic Diversity

Complete genotypes at 12 loci were determined for 277 individuals, and at 11 loci for 11 additional individuals (Appendix 3-I). Measures of genetic diversity were calculated from observed allele distributions and are presented in Table 3-1. Although the number of alleles per locus ranged from three (Locus BM5004) to 12 alleles (Locus BM4513), the average number of alleles per locus observed within most populations was between three and four. The Jasper population had the greatest average number of alleles. The average expected heterozygosity for a locus ranged from 0% to 79.55%, and average expected heterozygosities within populations ranged from 25.75% to 53.97%. The probability of identity ranged from 1 in 1.1×10^3 to 1 in 6.0×10^9 (Table 3-1). The Burwash herd had the lowest heterozygosity and highest probability of identity, while Yellowstone population had one of the highest heterozygosities and lowest probability of identity. The isolated population on Vancouver Island had an average expected heterozygosity of 34%, which was the second lowest value observed.

From 132 genotype distributions that could be examined using the Monte-Carlo approximation of the Fisher's exact test, nine and 19 genotype distributions were excluded because they had respectively, one and two alleles. Eleven of the genotype distributions were outside of the Hardy-Weinberg (HW) expectation at the 5 % level and 12 at the 1 % level. Given the number of tests performed, values out of HW equilibrium are expected. When the Dunn-Sidák experiment-wise error rate was applied (Sokal & Rohlf 1995), only genotype distributions for loci BM4208 and BM5004 from Banff, locus BM4513 from Vancouver Island, and locus RT13 from

Riding Mountain populations deviated from HW equilibrium at the 1% level. The deviations from the Monte-Carlo expectation were largely due to the presence of homozygotes for rare alleles. The 19 loci with two alleles were evaluated with the X^2 goodness- of - fit test, and only the genotype distribution for locus 5004 from Elk Island was rejected at the 5 % level. This population had an excess of heterozygotes.

Table 3-1. Measures of genetic diversity among wapiti populations as estimated using 12 microsatellite loci where H, is the expected heterozygosity; A is the number of alleles, and SE is the standard error.

Population	Mean H_E	SE H_E	Mean A	SE A	Overall probability of identity (1 in X)
Banff	0.4781	0.060	4.42	0.514	359 100 000
Burwash	0.2575	0.057	1.92	0.229	1 102
Elk Island	0.3981	0.058	2.92	0.358	5 957 200
French River	0.3410	0.056	2.25	0.241	444 730
Jasper	0.4742	0.027	5.00	0.492	32 607 000
Kootenay	0.5064	0.030	4.67	0.414	119 220 000
Riding Mt.	0.4907	0.036	4.17	0.423	693 590 000
Vancouver Is	0.3396	0.072	2.92	0.386	214 780
Ya-ha-tinda	0.5397	0.033	4.33	0.414	810 000 000
Yellowstone	0.5285	0.030	4.75	0.607	5 998 400 000
Yoho	0.4347	0.031	3.75	0.271	48 967 000

Relationships among populations

Nei's standard distances (Ds) and Nei's minimum distances (Dm) were estimated from the unbiased allele frequecies (Table 3-2). Ds distances ranged from -0.6947 to 0.0434 and Dm distances ranged from 0.3472 to 0.0186. The largest distances were to the Vancouver Island and the French River populations, while the smallest distances were between populations from Riding Mountain and Yellowstone National Parks. These distances were used to construct unrooted trees using both NJ and FM methods in the analysis package PHYLIP 3.672c. NJ trees derived from Dm and Ds distance measures and the FM tree derived from Ds distances produced trees with the same topology, therefore only the tree based on Ds measures is shown (Figure 3-3). The relationships among Jasper, Elk Island, French River, Burwash, Banff, Yoho, and Kootenay populations were retained in the FM tree based on Dm distances, however, the Vancouver Island wapiti grouped closer to the Yellowstone population and the position of the Ya Ha Tinda, Riding Mountain, and Yellowstone populations rotated counter-clockwise. Therefore, Ya Ha Tinda moved to the Riding Mountain branch location, Riding Mountain moved to the Yellowstone branch location, and Yellowstone moved to the Ya Ha Tinda branch location.

Table 3-2. Genetic distances among wapiti populations estimated from the number of shared alleles. Nei's standard distances (Ds) are given in the upper half and Nei's minimum distances (Dm) are given in the lower half.

	Source Population										
	BNP	BUR	EINP	FREN	JNP	KNP	RMNP	VAN	YAHA	YOHO	YNP
Banff	0.0	0.6245	0.2909	0.3959	0.1229	0.1031	0.1506	0.2581	0.1359	0.1069	0.1305
Bur	0.2932	0.0	0.3761	0.1001	0.3567	0.4180	0.3619	0.6947	0.4503	0.4054	0.6139
Elk Is.	0.1323	0.2107	0.0	0.2985	0.2089	0.1874	0.1974	0.4079	0.1938	0.1849	0.2135
French	0.1880	0.0684	0.1574	0.0	0.2527	0.2545	0.3067	0.5473	0.3229	0.2786	0.3417
Jasper	0.0554	0.1956	0.0998	0.1310	0.0	0.0709	0.0694	0.2290	0.0547	0.0458	0.1238
Koot	0.0452	0.2176	0.0890	0.1302	0.0321	0.0	0.0526	0.2063	0.062	0.0434	0.0528
Riding	0.0650	0.1961	0.0935	0.1519	0.0317	0.0231	0.0	0.2270	0.0544	0.0498	0.1208
Van. Is.	0.1298	0.3472	0.2004	0.2694	0.1178	0.1067	0.1158	0.0	0.1825	0.1822	0.2736
YaHa	0.0599	0.2319	0.0927	0.1594	0.0254	0.0279	0.0245	0.0978	0.0	0.0684	0.0833
Yoho	0.0461	0.2113	0.0873	0.1392	0.0213	0.0186	0.0216	0.0962	0.0280	0.0	0.0965
Yellow	0.0624	0.2998	0.1055	0.1730	0.0597	0.0270	0.0575	0.1407	0.0410	0.0469	0.0

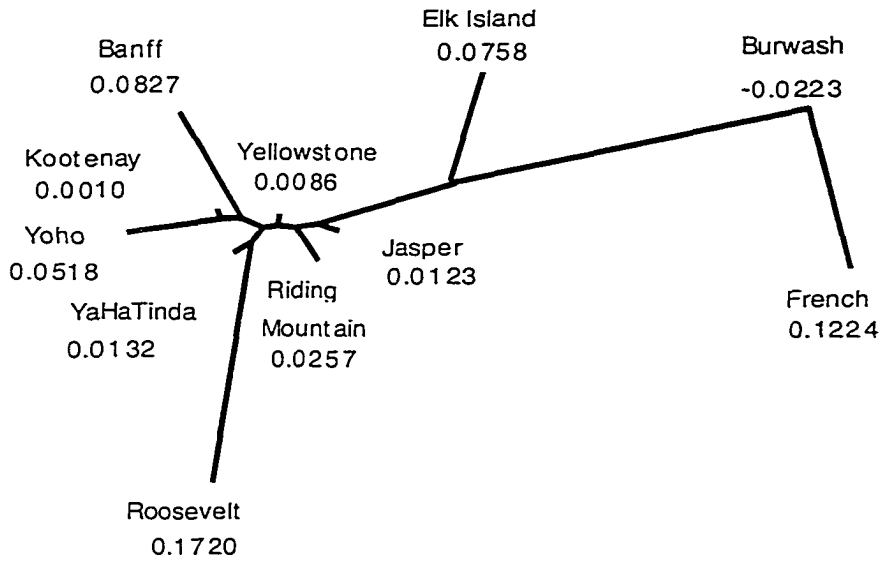

Figure 3-3. An unrooted NJ tree constructed from Nei's standard genetic distances for eleven populations of wapiti. Terminal branch lengths to populations are noted.

How distinct populations are from each other can be inferred from the assignment test, although the power of the assignment test is proportional to the number of loci included in the study. A total of 191 individuals from 288 (67.5 %) were assigned to their correct populations (Table 3-3). Although all Vancouver Island wapiti were correctly assigned, only 37 % of Kootenay and 36 % of Yellowstone animals were assigned to their respective herds. The low frequency of observing individuals from

another population at the same or greater likelihood in the randomization tests indicated that many misassignments were unlikely to be due to chance. For example, the assignment of three or more individuals from Burwash to French River occurred in only 0.006 of the randomization replicates. Similarly, the assignment of an equal or greater number of individuals from Yoho to Banff or Kootenay occurred, respectively, in only 0.039 and 0.050 of the randomization replicates.

Table 3-3. The numbers of individuals assigned to each population using the assignment test. Individuals correctly assigned to their source population are shown in bold. The randomization test was used to infer which observations were unlikely to occur from the random combination of alleles, and observations that occur with low frequencies (Rdf) in 1000 replicates are noted with an asterisk (*) when Rdf < 0.05 and with a cross (†) when Rdf< 0.25.

Source		Populations to which individuals are assigned										
Population	2N	BNP	BUR	EINP	FRE	JNP	KNP	RMNP	VAN	YENP	YONP	YAHA
Banff	28	**24**	0	0	0	1†	1†	0	0	1†	0	1†
Burwash	14	0	**11**	0	3*	0	0	0	0	0	0	0
Elk Island	20	0	0	**17**	1*	0	0	0	0	1*	1*	0
French River	10	0	1	0	**8**	0	0	1*	0	0	0	0
Jasper	56	0	0	1	1†	**35**	1	1	0	7*	1	9*
Kootenay	27	0	0	0	0	2	**10**	3	1†	3	4†	4†
Riding Mt.	36	0	0	2†	0	3	1	**23**	1†	5	0	1
Vancouver Is.	30	0	0	0	0	0	0	0	**30**	0	0	0
Yellowstone	22	0	0	0	0	4†	3	5†	0	**8**	0	2
Yoho	15	1*	0	0	0	0	4*	0	0	0	**9**	1
YaHaTinda	30	0	0	1	0	3	1	4†	0	1	4†	**16**

The log likelihoods that individuals from two different populations would assign to either population were plotted (Figure 3-4) using the program Delta Graph and the numbers of individuals that misassigned were noted in Table 3-4. The scatter plots generated indicate the relative amount of relatedness among the population, where a population of closely related individuals would be represented by a tight cluster. Once again, the number of loci employed can influence the degree of clustering. The amount of overlap between two populations is a measure of the differentiation between populations where populations that have significantly diverged from each other would be represented by two distinct genotype distributions e.g. Figure 3-4a. The presence of a misassigned individual in a comparison of two distinct populations suggests the individual is a migrant, whereas overlapping genotype distributions e.g. Figure 3-4b suggest populations have not diverged from each other or they share migrating animals (Waser & Strobeck 1998). Surprisingly, Riding Mountain and Yellowstone populations

are separated by more than a thousand kilometers and do not share recent founders (<100 years), but have nearly indistinguishable genotype distributions.

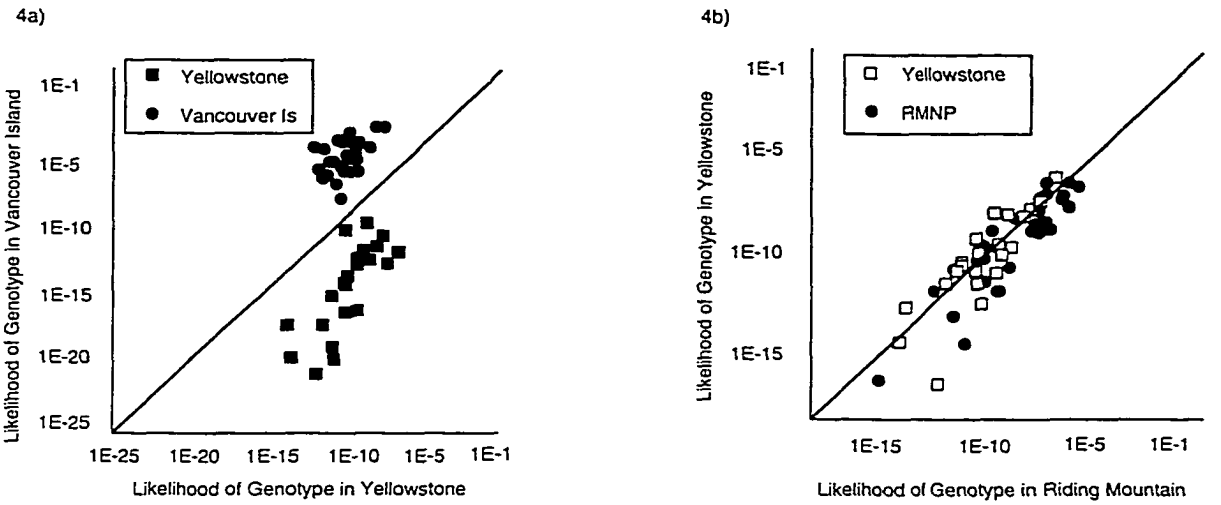

Figure 3-4. Genotype distributions as indicated from the likelihood estimates that they would appear in the source and other populations. a) The assignment of individual genotypes from Vancouver Island and Yellowstone National Park to both populations using the probability the genotypes will occur in each population. b) The assignment of individual genotypes from Riding Mountain and Yellowstone National Park to both populations using the probability the genotypes will occur in each population.

Another perspective of a population's distinctiveness can be made from a comparison of the proportion of each population that is misassigned to a source population. Banff and Burwash populations both have individuals misassigned to them from two populations (Table 3-4). However, the proportion of individuals misassigned to Banff comprised 10 and 20 % of Elk Island and Yoho populations while individuals misassigned to Burwash comprised only 10 and 4 % of French River and Kootenay populations. The fewest individuals misassigned to Vancouver Island and Burwash populations, and therefore they represent the most distinct populations. Populations that were the least differentiated from other populations were from Ya Ha Tinda, Riding Mountain, and Yellowstone.

Allele sharing distances were calculated among the 56 individuals from Jasper National Park, and a NJ tree was constructed (Figure 3-5), where individuals numbers correspond to those noted in Figure 3-2. Relatedness between individuals ranged from 0.24 to 0.91, with a population average of 0.57. Thirty-eight of the 56 individuals that were identified as possible sibling or parent/offspring pairs were often found in different locations while distantly related individuals were often found in the same location. A

similar observation was reported with the assignment test where 22 of the 28 wapiti located on the west side of Jasper were assigned to the east side, and 13 of the 28 wapiti found on the east side of Jasper were assigned to the west side (Figure 3-5).

Table 3-4. The distribution of individuals misassigned in comparisons between two populations. Because source populations cannot be misassigned to their own population, these comparisons are indicated by a '-'. The total of the misassigned individuals between two populations will indicate the number of overlapping genotypes between the populations. The proportion of each source population that was misassigned to another population was totaled (Σ) and was used to illustrate how common the genotypes are to each population.

Source	Number of individuals that were misassigned in pair-wise comparison										
Population	BNP	BUR	EINP	FRE	JNP	KNP	RMNP	VAN	YENP	YONP	YAHA
Banff	-	0	2	0	4	3	3	0	4	2	3
Burwash	0	-	0	2	0	0	0	0	0	0	0
Elk Island	2	0	-	2	2	2	1	1	2	2	2
French River	0	1	1	-	1	0	1	0	1	1	1
Jasper	0	0	1	1	-	5	7	0	10	2	12
Kootenay	0	1	1	1	5	-	6	1	11	8	7
Riding Mt.	0	0	2	0	3	4	-	1	10	1	6
Vancouver Is.	0	0	0	0	0	0	0	-	0	0	0
Yellowstone	0	0	0	0	7	6	11	0	-	4	6
Yoho	3	0	1	0	2	6	3	0	3	-	3
YaHaTinda	0	0	1	0	8	5	7	1	5	6	-

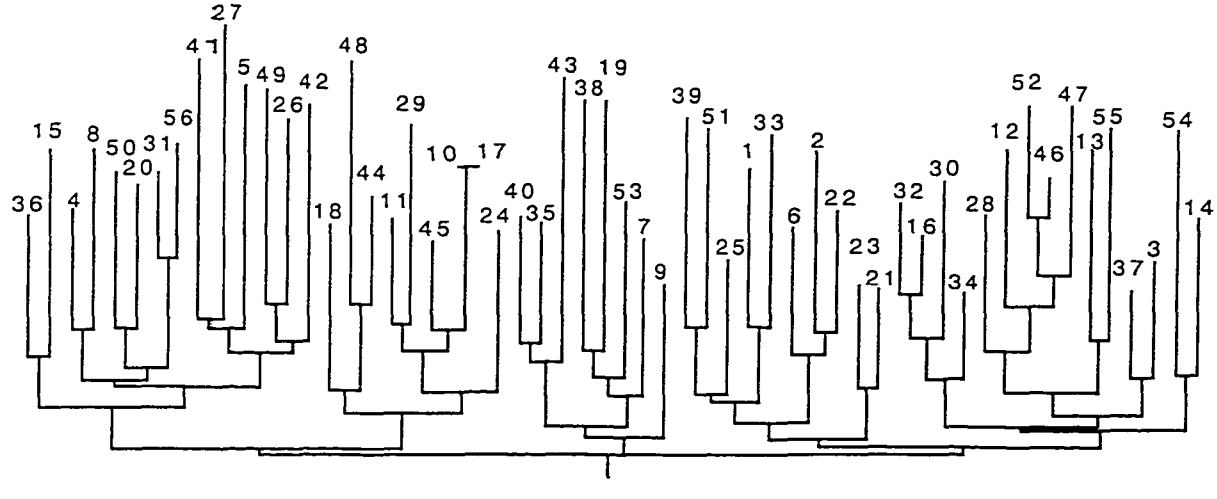

Figure 3-5. A NJ tree constructed from allele sharing distances for 56 individuals from Jasper National Park. The numbers correspond to the sample locations identified in Figure 3-2.

Discussion

Diversity within the species

Seton estimated 10,000,000 wapiti could be found on the North American continent prior to the arrival of Europeans and by the start of the 1900's they were reduced to between 50,000 - 100,000 animals with up to 40,000 animals found in the Yellowstone area (Murie 1951). Little genetic diversity appears to have been lost in this population decline as suggested by the overlapping distribution of genotypes from the large indigenous populations in Riding Mountain, and Yellowstone National Parks, which in turn hints a low level of diversity prior to the population decline. A low level of genetic diversity is consistent with the observation of few alleles in many microsatellite loci surveyed in North American *Cervus elaphus* (Talbot *et al.* 1996; Wilson *et al.* 1997; Roed & Midthjell 1999).

Although a preoccupation exists to explain low genetic variation by bottleneck effect (Amos & Harwood 1998), the choice of a microsatellite itself has a major impact on the measurement. A survey of European red deer (*C. elaphus*) identified several markers with more than ten alleles per locus (Slate *et al.* 1998) which suggests choice of loci may be a reason for the reduced measure of diversity. Although wapiti surveyed at four of the same loci had both equal or greater variation, heterozygosity estimates may have been higher had more variable loci been included. Reduced microsatellite variability, such as observed in loci BM5004 and ETH 152, also may be due to low mutation rate (Hendrick & Parker 1997) or size constraint (Paetkau *et al.* 1997). However, given that most studies will be biased for selecting only informative loci, the genetic diversity of North American wapiti at present appears to be greater than that observed for bison (Wilson & Strobeck 1999) and moose (Broders *et al.* 2000) and considerably less than that observed for caribou (Zitlau *et al.* in press).

Diversity among subspecies

Current taxonomic divisions for *C. elaphus* ssp. are described by Bryant and Maser (1982). However they state that only three of the six named subspecies (Roosevelt, Tule, and Merriam) meet the criterion of isolation necessary for the evolution of genetically distinct biological subspecies. A distinction between the Rocky Mountain and Manitoban subspecies has little support from protein electrophoretic analysis (Glenn & Smith 1993), mtDNA phylogenies (Cronin 1992, Polziehn *et al.* 1998), or morphological comparisons (Schonewald 1994; Hutton 1972; Blood & Lovaas 1966). Populations appear to be ascribed to subspecies that fall within

ecological zones where no obvious behavioral, social, or geographical barriers are observed. Glenn and Smith (1993) noted a slight difference between Roosevelt and Rocky Mountain populations, and Polziehn *et al.* (1998) identified unique mitochondrial lineages for Roosevelt and Tule animals. A preliminary microsatellite analysis comparing Tule, Rocky Mountain and Manitoban wapiti also differentiated the Tule population (Denome 1998). Microsatellite analysis from this study supports the subspecific status of *roosevelti* for wapiti on Vancouver Island, British Columbia and the overlapping genotype distributions of indigenous Manitoban and Rocky Mountain subspecies supports the need to re-examine the classification of these subspecies. While microsatellite loci on their own cannot identify subspecies, the technique is useful for describing the genetic relationships among populations and can identify isolated populations.

Furthermore, no support was found in this study for the existence of any Eastern wapiti descendants. Because the French River and Burwash herds exhibited alleles common to all populations and did not harbor any unique alleles, either the Eastern wapiti were not distinct from other subspecies or no descendants of the original populations supplemented these two herds. The French River and Burwash herds, located in the historic range of the Eastern wapiti, also had no unique mtDNA lineages to support the presence of the Eastern subspecies (Polziehn *et al.* 1998).

Although the similar distribution of alleles in Rocky Mountain and Manitoban animals may lead one to believe that early relocations of animals have had little effect on the genetic diversity of local populations, differentiation can occur in adaptive traits but show little genetic structure at neutral genetic loci (Karhu *et al.* 1996). Therefore, wildlife managers should consider morphological, ecological, and genetic differences when relocating animals.

Diversity within and among populations

A small number of founders and geographic isolation are factors that have contributed to the reduction of diversity in populations from Burwash, Elk Island, French River, and Vancouver Island. For example, from 1932 to 1934, between 24 and 75 wapiti from Buffalo Park, Wainwright, Alberta were introduced to the Burwash Industrial Prison near Sudbury, Ontario, and the population grew to an estimated 300-400 animals by 1949 (Stelfox 1993). After a giant liver fluke scare, most animals in the enclosure were slaughtered, although some wapiti had already escaped through damaged fences and more were released by prison guards opposed to the slaughter. The wapiti dispersed into the surrounding area and southeast to French River. About 150

free-ranging wapiti were in the area by the 1950's, which gave rise to the belief that indigenous Eastern wapiti still may exist. Once again wapiti in Ontario were hunted until near extinction and by 1980, when hunting was banned, only 30 animals found in French River and Burwash were thought to have survived. Because these herds share a small number of founders, and add up to no more than a total of 100 animals, not surprisingly they have the lowest genetic diversity.

Vancouver Island has supported a population of wapiti that potentially has been isolated from the mainland for an estimated 7000 years (Pielou 1991). Although wapiti can swim between islands (Spalding 1992), the low heterozygosity suggests it is unlikely animals have recently migrated to Vancouver Island and there are no records of exchanges or relocations of wapiti having taken place. Immigration from a large source population can strikingly slow, halt, or reverse the loss of genetic variation, even with only one or a few migrants per generation (Lacy 1987). The population was also restricted to approximately 200 animals in 1913 and possibly may have been lower prior to this estimate (Spalding 1992). The occurrence of low genetic diversity is common to mammal populations on islands (Frankham 1997), and currently it is impossible to deduce the combination of factors have led to the low genetic diversity in the Vancouver Island population.

A reduction of genetic diversity will occur in all populations, but the rate of loss can be expedited in small or isolated populations unless gene flow is sufficiently high (Amos & Harwood 1998). The island and fragmented populations of wapiti from Burwash, Elk Island, French River, or Vancouver Island have heterozygosities that are comparable to other mammal populations that have undergone population declines (Paetkau *et al.* 1998, and papers within; Wilson & Strobeck 1999). No visible abnormalities have been reported within these populations of wapiti, and if the number of wapiti relocated from Elk Island National Park is an indication of fecundity (Stelfox 1993), wapiti do not appear to suffer from their decreased genetic variability. However, the long-term survival of small or isolated populations cannot be predicted even if they appear at present to be prospering. Regular supplementation of animals to small populations has been suggested as a means of increasing or maintaining diversity (Lacy 1987).

The current distribution of genetic diversity is in part a function of gene flow that can be restricted by natural barriers and anthropogenic deterrents (Amos & Harwood 1998). The distribution of populations within the Rocky Mountains appears to be a continuum and indeed the distribution of genotypes suggests animals are traveling between some of the corridors connecting the parks. Wapiti were uncommon

to the interior ranges of the Rocky Mountains prior to the early 1900's, and during the early 1970's, animals from Banff were released into the Rocky Mountain trench near the Kootenay National Park border (Spalding 1992). While founder effect and migration may explain the large number of Banff animals that assigned to the Kootenay population, genetic distances and genotype distributions suggest that the movement of wapiti dispersing from Yoho and Kootenay into Banff, also observed from tagged animals (Gibbons 1978), is far from extensive. In contrast, the genotype distributions from Jasper and Banff are distinct, which suggests gene flow is restricted between the two parks. Although north-south corridors connect Jasper and Banff, the inhospitable terrain (ice fields) and an absence of suitable habitat near the park boundaries may discourage the movement of animals. Among isolated populations the number of births and deaths may play a more important role in maintaining local genetic diversity whereas in continuous populations migrants may have more impact (Amos & Harwood 1998).

Geographic and habitat features are unlikely to explain the separation between the Banff and Ya Ha Tinda herds. The Ya Ha Tinda herd is found near the east boundary of Banff and was likely established from wapiti that dispersed from Banff in the 1930's (Morgantini 1995). The current separation between the two populations, as suggested by the assignment test, is probably enforced by the presence of wolves. The re-establishment of wolves around Banff was suggested to have concentrated wapiti seeking safety into the town of Banff (Kay *et al.* 1994).

Determining the success of relocated animals can be achieved by monitoring using radio collars and ear tags, but the assignment test may offer an additional method of identifying these animals (Waser & Strobeck 1998). Like migrants, relocated animals should have a greater probability of assigning to their founding population (Paetkau *et al.* 1995). Although wapiti from Elk Island National Park have been released into areas near National Parks (Spalding 1992, Stelfox 1993), few animals have misassigned to Elk Island. This may suggest the transplanted animals are establishing themselves in new territories, however, the pair-wise comparison suggests slightly less differentiation between populations and the need to improve discriminating power by including more loci.

Defining a population or a herd can be useful for management applications, however artificial boundaries such as park borders and congregations of animals in the same location often serve to delimit the distribution of a population or herd. Moritz (1994) suggested management units be identified as regions where populations have significantly different genotype distributions. When genotypes of wapiti were used to

determine relatedness among individuals and probability of assignment, unrelated and related individuals were found in the same area. This suggests that there is little fidelity of some animals to a herd. Woods (1991) found wapiti populations in Banff consisted of migrating and resident animals, and personal observations of collared wapiti within Jasper also suggested movement among herds. The high density of wapiti along the Athabasca-Miette River valley and wide representation of age groups within the established population of Jasper, support Albon *et al's.* (1992) observations that suggest familial members were likely to disperse under these circumstances. Therefore, from a management perspective, wapiti found within the boundaries of Jasper appear to act as a panmictic population.

Managing populations to maintain genetic diversity requires an understanding of the factors that shape genetic distances among populations, such as predator distribution, habitat suitability, and geographic layout. Genetic diversity can also be used to identify populations that may benefit from the addition of relocated animals or that should remain status quo because they represent different subspecies.

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Appendix 3-1. Number of copies of each allele, unbiased expected heterozygosity (H), and probability of identity (I) per locus in each population of wapiti.

Locus RT13	Number of Alleles							
Park	2N	297	299	303	305	307	H	I
Banff	56	0	0	0	56	0	0	1
Burwash	28	0	0	0	28	0	0	1
Elk Island	40	0	0	0	40	0	0	1
French River	20	0	0	0	20	0	0	1
Jasper	112	1	9	9	92	1	0.597	0.250
Kootenay	52	0	1	1	49	1	0.646	0.177
Riding Mt	72	2	0	12	58	0	0.327	0.485
Vancouver Is	60	7	0	0	53	0	0	1
YaHaTinda	60	2	4	1	41	4	0.372	0.400
Yellowstone	44	0	3	1	37	3	0.740	0.110
Yoho	30	0	0	3	26	1	0.480	0.301

Locus BL42		Number of Alleles							
Park	2N	248	250	256	258	260	264	H	I
Banff	56	0	33	0	14	9	0	0.575	0.242
Burwash	28	0	5	0	23	0	0	0.304	0.515
Elk Island	40	0	28	0	12	0	0	0.431	0.411
French River	20	0	3	0	17	0	0	0.268	0.547
Jasper	112	0	70	1	24	17	0	0.545	0.261
Kootenay	54	0	19	0	18	15	2	0.700	0.154
Riding Mt	72	0	40	1	26	5	0	0.564	0.275
Vancouver Is	60	0	30	0	8	22	0	0.608	0.234
YaHaTinda	60	0	34	0	19	7	0	0.575	0.256
Yellowstone	44	0	27	0	11	6	0	0.555	0.257
Yoho	30	2	12	0	10	6	0	0.708	0.142

Locus BM1009		Number of Alleles							
Park	2N	284	286	288	290	280	272	H	I
Banff	56	2	44	8	0	1	1	0.367	0.420
Burwash	28	28	0	0	0	0	0	0	1
Elk Island	40	2	21	0	0	17	0	0.555	0.302
French River	20	12	7	0	1	0	0	0.542	0.300
Jasper	112	8	91	7	5	0	1	0.332	0.456
Kootenay	54	12	38	1	1	1	1	0.463	0.333
Riding Mt	72	15	51	2	1	3	0	0.469	0.335
Vancouver Is	60	2	30	28	0	0	0	0.540	0.324
YaHaTinda	60	2	51	4	1	0	2	0.275	0.526
Yellowstone	44	7	27	7	1	2	0	0.584	0.206
Yoho	30	1	22	0	3	0	4	0.448	0.318

Locus BM888	Number of Alleles							
Park	2N	179	187	189	193	185	H	I
Banff	56	34	20	2	0	0	0.512	0.334
Burwash	28	3	25	0	0	0	0.198	0.647
Elk Island	40	12	20	8	0	0	0.636	0.203
French River	20	13	7	0	0	0	0.479	0.380
Jasper	112	56	47	1	8	0	0.574	0.274
Kootenay	54	30	19	4	0	1	0.572	0.263
Riding Mt	72	28	42	2	0	0	0.515	0.340
Vancouver Is	60	0	48	11	0	1	0.332	0.485
YaHaTinda	60	16	36	5	3	0	0.569	0.253
Yoho	30	21	8	1	0	0	0.663	0.179
Yellowstone	44	19	17	4	4	0	0.453	0.364

Locus BM5004	Number of Alleles					
Park	2N	133	135	137	H	I
Banff	28	3	14	11	0.606	0.242
Burwash	28	21	0	7	0.389	0.438
Elk Island	40	16	0	24	0.492	0.378
French River	20	13	0	7	0.479	0.380
Jasper	112	67	1	44	0.492	0.370
Kootenay	54	15	0	39	0.409	0.428
Riding Mt	72	21	0	51	0.419	0.422
Vancouver Is	60	9	0	51	0.260	0.588
YaHaTinda	60	31	0	29	0.508	0.375
Yellowstone	44	18	0	26	0.495	0.377
Yoho	30	12	1	17	0.536	0.323

Locus ETH152	Number of Alleles							
Park	2N	191	193	195	197	199	H	I
Banff	56	11	8	7	16	14	0.795	0.074
Burwash	28	2	0	24	2	0	0.265	0.537
Elk Island	38	8	0	10	20	0	0.626	0.206
French River	18	4	0	14	0	0	0.366	0.446
Jasper	110	49	0	26	35	0	0.650	0.196
Kootenay	54	17	0	12	23	2	0.681	0.165
Riding Mt	72	13	0	16	43	0	0.569	0.244
Vancouver Is	60	51	0	8	1	0	0.264	0.561
YaHaTinda	60	20	0	5	34	1	0.570	0.261
Yellowstone	36	14	0	9	13	0	0.675	0.180
Yoho	30	6	0	1	23	0	0.384	0.416

Locus RT1		Number of Alleles									
Park	2N	216	218	220	224	226	228	230	232	212	H	I
Banff	56	0	36	2	9	0	0	9	0	0	0.544	0.247
Burwash	28	0	2	10	0	0	0	16	0	0	0.561	0.280
Elk Island	40	0	30	0	2	2	0	6	0	0	0.421	0.354
French	20	0	6	13	1	0	0	0	0	0	0.511	0.317
Jasper	112	0	50	0	11	0	1	50	0	0	0.597	0.250
Kootenay	54	0	28	1	7	0	0	15	3	0	0.646	0.177
Riding Mt	72	2	28	1	4	0	0	34	1	2	0.629	0.207
Vancouver Is	60	0	60	0	0	0	0	0	0	0	0	1
YaHaTinda	60	0	35	5	6	0	0	13	1	0	0.606	0.196
Yellowstone	44	0	17	0	8	0	2	13	1	3	0.740	0.110
Yoho	30	0	21	1	6	0	0	2	0	0	0.480	0.301

Locus BM203		Number of Alleles								
Park	2N	221	223	225	227	229	231	233	235	H	I
Banff	56	5	0	10	20	20	1	0	0	0.718	0.131
Burwash	28	0	0	0	0	28	0	0	0	0	1
Elk Island	40	2	0	6	0	32	0	0	0	0.343	0.452
French	20	0	0	1	3	16	0	0	0	0.353	0.425
Jasper	112	7	1	1	39	62	2	0	0	0.573	0.260
Kootenay	54	7	0	8	21	13	3	0	2	0.762	0.090
Riding Mt	72	8	0	19	25	15	5	0	0	0.760	0.097
Vancouver Is	60	0	0	13	43	4	0	0	0	0.442	0.359
YaHaTinda	60	0	6	1	4	35	13	1	0	0.608	0.192
Yellowstone	44	4	0	12	10	14	3	1	0	0.777	0.086
Yoho	30	1	0	2	18	8	1	0	0	0.582	0.223

Locus 193		Number of Alleles										
Park	2N	106	116	120	122	124	128	140	142	146	H	I
Banff	56	0	3	1	38	1	1	1	0	11	0.506	0.276
Burwash	28	0	0	0	28	0	0	0	0	0	0	1
Elk Island	40	0	9	0	31	0	0	0	0	0	0.358	0.485
French River	20	0	0	0	20	0	0	0	0	0	0	1
Jasper	112	0	6	1	85	4	1	4	1	10	0.414	0.354
Kootenay	54	2	4	0	33	4	1	0	0	10	0.590	0.196
Riding Mt	72	0	2	0	51	0	3	0	0	16	0.453	0.347
Vancouver Is	60	0	1	0	58	0	0	0	1	0	0.066	0.870
YaHaTinda	60	0	10	0	37	0	0	2	2	7	0.582	0.203
Yellowstone	44	0	1	0	31	0	1	1	0	10	0.461	0.336
Yoho	30	0	1	0	22	0	0	0	0	7	0.421	0.386

Locus BM4107		Number of Alleles										
Park	2N	157	159	161	163	165	167	169	171	173	H	I
Banff	56	0	33	1	0	0	0	2	20	0	0.533	0.308
Burwash	28	0	4	0	0	0	0	4	20	0	0.466	0.312
Elk Island	40	0	20	0	0	0	10	10	0	0	0.641	0.197
French River	20	0	8	0	0	0	0	0	12	0	0.505	0.371
Jasper	112	4	43	1	1	1	1	10	51	0	0.641	0.196
Kootenay	54	0	27	0	0	0	0	6	21	0	0.597	0.248
Riding Mt	72	0	23	0	0	3	0	9	32	5	0.688	0.149
Vancouver Is	60	5	25	0	0	0	1	15	2	12	0.728	0.116
YaHaTinda	60	0	30	0	0	0	0	1	29	0	0.525	0.346
Yellowstone	44	0	28	0	0	0	0	4	11	1	0.536	0.268
Yoho	30	0	22	0	0	0	0	3	5	0	0.439	0.341

Locus BM4513		Number of Alleles													
Park	2N	114	118	124	126	128	130	132	134	136	138	140	144	H	I
Banff	56	0	0	0	4	4	1	38	6	2	0	0	1	0.525	0.237
Burwash	28	0	0	0	14	0	14	0	0	0	0	0	0	0.519	0.367
Elk Island	40	0	7	1	27	1	1	3	0	0	0	0	0	0.519	0.254
French River	20	0	0	2	0	6	0	10	2	0	0	0	0	0.674	0.156
Jasper	112	0	0	0	18	2	13	69	5	0	5	0	0	0.582	0.205
Kootenay	54	0	0	0	8	6	3	26	6	3	0	0	2	0.727	0.095
Riding Mt	72	0	5	0	24	4	6	32	1	0	0	0	0	0.686	0.150
Vancouver Is	60	0	0	0	5	8	13	34	0	0	0	0	0	0.618	0.190
YaHaTinda	60	0	0	2	18	2	8	26	2	2	0	0	0	0.712	0.126
Yellowstone	44	1	1	1	9	2	3	22	3	0	1	1	0	0.710	0.105
Yoho	30	0	0	0	3	7	1	15	2	2	0	0	0	0.700	0.120

Locus BM4208		Number of Alleles											
Park	2N	142	144	146	148	150	154	156	158	162	164	H	I
Banff	56	0	1	0	0	12	11	1	16	0	15	0.775	0.090
Burwash	28	0	0	0	0	21	7	0	0	0	0	0.389	0.438
Elk Island	40	0	0	0	0	38	1	0	0	0	1	0.099	0.808
French River	20	0	0	0	0	17	3	0	0	0	0	0.268	0.547
Jasper	112	2	0	3	0	54	53	0	0	0	0	0.547	0.311
Kootenay	54	4	0	0	3	33	12	0	0	2	0	0.578	0.217
Riding Mt	72	0	0	0	5	33	34	0	0	0	0	0.570	0.284
Vancouver Is	60	0	0	0	0	20	40	0	0	0	0	0.452	0.400
YaHaTinda	60	0	0	0	5	31	24	0	0	0	0	0.525	0.272
Yellowstone	44	2	0	0	3	19	19	0	1	0	0	0.634	0.207
Yoho	30	1	0	0	2	23	4	0	0	0	0	0.402	0.366

Chapter 4

Phylogeny of wapiti, red deer, sika deer, and other North American cervids as determined from mitochondrial DNA[1]

Abstract

Red deer (*Cervus elaphus*) are divided into three subspecific groups; the first group includes seven subspecies from Europe and northern Africa; the second group includes seven subspecies from central Asia; and the third group includes nine subspecies from eastern Asia, Siberia, and North America. Recognition of the North American wapiti as a species has been denied on the basis of morphological similarity with red deer and the circumpolar distribution of *C. elaphus*. Sika deer (*C. nippon*), which is also distributed in Eastern Asia, shares phenotypic and genotypic similarities with *C. elaphus*. A comparison of sequences from the control region of mitochondrial DNA (mtDNA) from North American and Siberian wapiti, European red deer, and Asian sika deer, was used to construct a phylogenetic relationship among these cervids, and to other cervids found within North America; including white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*O. hemionus columbianus*), moose (*Alces alces*), and caribou (*Rangifer tarandus*). The mtDNA sequence divergence between wapiti and red deer was 5.60%, between wapiti and sika deer 5.19 %, and between sika deer and red deer 5.02 %, suggesting that the subspecies status of North American wapiti should be reviewed. The mtDNA sequence divergence between white-tailed deer and black tailed deer was 7.82 %, and is consistent with earlier mtDNA studies in *Odocoileus*.

Introduction

The North American elk or wapiti (*Cervus elaphus* subspecies), observed by European explorers in the 1500's, were likely named after the European elk (*Alces alces* or moose) due to a resemblance in size. Although "elk" is more widely used in North America, this common name has led to confusion at an international level, therefore the term wapiti will be used. Confusion has also resulted from failing to distinguish the North American wapiti from the European red deer (*C. elaphus*). Furthermore, Geist (1987),

[1] A version of this text was published. Polziehn RO, Strobeck C. (1998) *Molecular Phylogenetics and Evolution*, **10**, 249-258

suggested the sika deer (*C. nippon*), red deer (*C. elaphus*), and wapiti (*C. e. sibericus - nelsoni*) represent an oversimplified cline, which has led to much discussion on the possible relationship among these three species.

As early as 1737, the North American wapiti were accepted as a distinct form from the European red deer (Bryant and Maser 1982). However, Erxleben shortly thereafter described the Eastern wapiti (*C. e. canadensis* 1777) in North America as a subspecies of red deer (Linnaeus 1758). The North American wapiti was again raised to the species level *C. canadensis* in 1780 by Borowski (Bryant and Maser 1982). The ability to interbreed and the circumpolar distribution led Ellerman and Morrison-Scott (1951) and Flerov (1952) to remove the wapiti's status as a separate species, and at present the wapiti in North America are regarded as subspecies of *C. elaphus* (red deer). Cockerill (1984) and Whitehead (1972) continued to regard *C. elaphus* and *C. canadensis* as distinct species, with the border between the two groups to be drawn along the Tien Shan Mountains and the Gobi desert. Groves and Grubb (1987) recognized three general systematic groups for red deer (see Figure 4-1). The nominate group is divided into subspecies that occupy Europe, North Africa, and south western Asia. The wallachi group includes subspecies that are found in the Soviet Union, Afghanistan, Tibet, Bhutan, and north central China, all of which are listed on CITES appendices. The canadensis group includes the North American wapiti and unclassified populations in Manchuria, Mongolia, Korea, and southern Siberia, a region also occupied by the sika deer (*C. nippon* Temminck 1836). Lowe and Gardiner (1975) found several subspecies of sika deer could not be distinguished from red deer, which suggests a revision in their classification also may be necessary, and that sika subspecies found on the Japanese islands appear to be discontinuous from the mainland groups which form a natural continuum. The phylogenetic study of total mtDNA of sika deer also did not reflect the present taxonomic status of sika deer, which is based on morphology (Tamate and Tsuchiva 1995).

In early records of American natural history, Brickell stated, "Some take elk (wapiti) for the red deer of America, but I am credibly informed, that they are of two different kinds, and that they will never breed together (Bryant and Maser 1982: 4). Inability to interbreed would certainly support the recognition of specific status for wapiti. However, wapiti were shown to breed with red deer in New Zealand. The fertile hybrids led to support for a conspecific status of red deer and North American wapiti, although the extent to which the hybrids contributed to the population is unknown. North American wapiti and red deer are also able to produce fertile hybrids with a number of related species including the sika deer (Lowe and Gardiner 1975, Abernathy 1994) and Pere David's deer (*Elaphurus davidianus*; Tate *et al.* 1995).

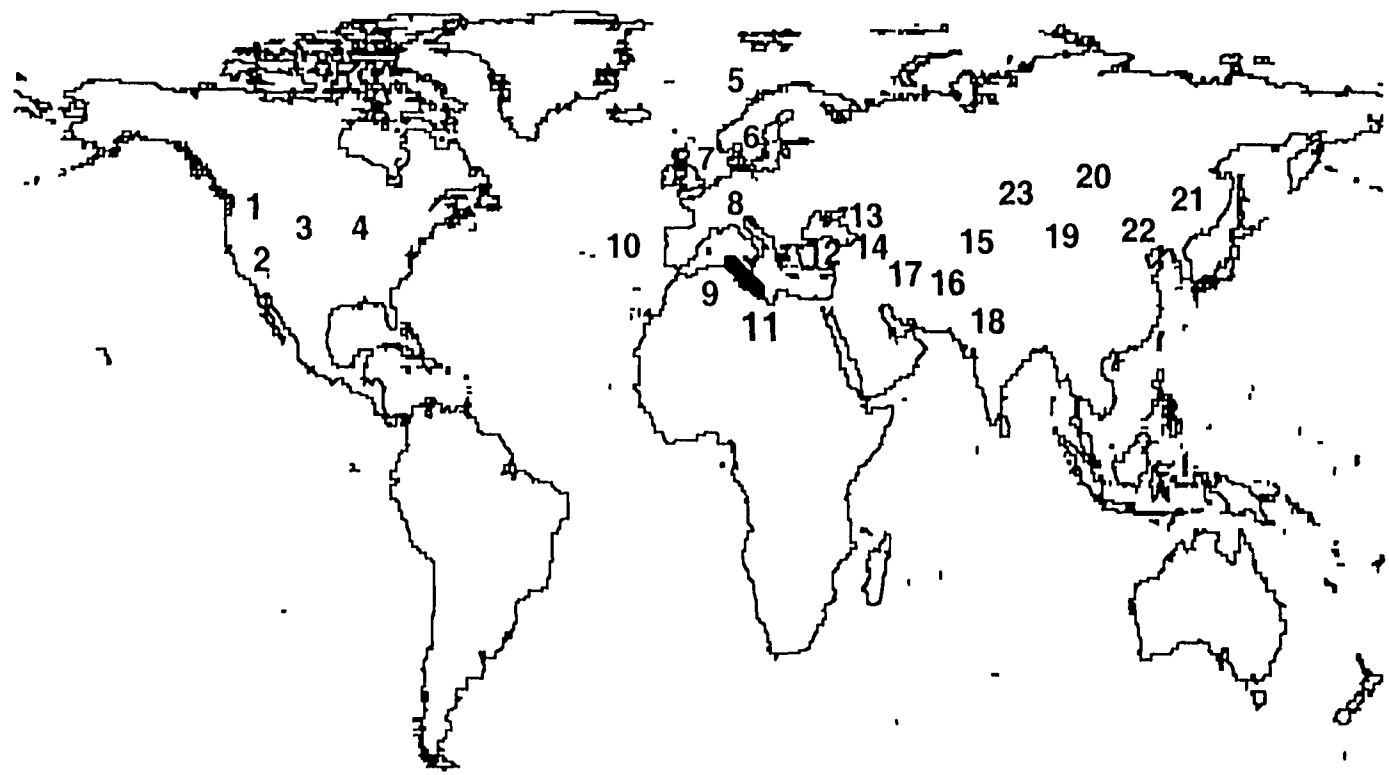


Figure 4-1. A biogeographical distribution of the three groups of *Cervus elaphus* subspecies as adapted from Groves and Grubb (1982) and Ellerman and Morrison-Scott (1951). The European group consists of subspecies 5 - 11, the central Asian group consists of subspecies 12 - 19, and the east Asian and North American group consist of 20 - 23 and 1 - 4. 1 *C. e. roosevelti*, 2 *C. e. nannodes*, 3 *C. e nelsoni*, 4. *C. e. manitobensis*, 5 *C. e. atlanticus*, 6 *C. e. elaphus*, 7 *C. e. scoticus*, 8 *C. e. hippelaphus*, 9 *C. e. barbarus*, 10 *C. e. hispanicus*, 11 *C. e. corsicanus*, 12. *C. e. maral*, 13 *C. e. bactrianus*, 14 *C. e. yarkandensis*, 15 *C. e. wachei*, 16 *C. e. hanglu*, 17 *C. e. wallichi*, 18 *C. e. macneilli*, 19 *C. e. kansuensis*, 20 *C. e. asiaticus/sibericus*, 21 *C. e. xanthopygus*, 22 *C. e. alashanicus*, 23 *C. e. songaricus*

Red deer and wapiti, although similar in appearance, can be distinguished from one another. Red deer weigh about 100-200 kg, measure 165-250 cm in length and 120-150 cm tall at the shoulder, and are reddish brown. In comparison, wapiti weigh about 200-400 kg, measure 225-250 cm in length and 140-150 cm tall at the shoulder, and are grayish brown. Red deer have longer tails, less defined rump patches, and more crownlike antlers. Sika deer are slightly smaller, weighing about 80 kg, measuring 95-180 cm in length and 65-109 cm at the shoulder, with a well defined rump patch (Geist 1982). Male red deer produce deep guttural roar during mating, while wapiti bulls have high pitched bugles (Tembrock 1965). Male and female wapiti are less dimorphic than red deer, as both sexes have throat manes, and have a weight ratio of males to females at 1.19-1.38 (Smith 1974). Both sika deer sexes have manes in winter, and they have a weight ratio of 1.60-1.74 (Kiddie 1962, Nowak 1991). Red deer have a ratio of 1.54-2.10 (Beninde 1937), and only males have throat manes. Geist (1982) believed antler complexity, rump patch, tail configuration, body size, social behavior and the voice of rutting stags changed with changes in the environment, and that they are reflected most strongly in the populations at opposite ends of a cline.

Most genetic studies focus on the variation within European red deer (Herzog *et al.* 1991, Hartl *et al.* 1995). While Johnson (1968) was unable to distinguish red deer from wapiti using serum proteins or hemoglobin, Dratch (1986) noted that nearly all red deer exhibited one type of hemoglobin and wapiti another. Dratch (1986) made a similar observation for the SOD protein, although a small proportion of wapiti had both types. Although protein electorphoresis revealed marked differences between red deer and wapiti (Baccus *et al.* 1983, Dratch and Gyllensten 1985) these differences were not absolute. Similar observations were made when comparing the transferrin protein between sika deer and red deer (McDougall and Lowe 1968). The amount of variation in proteins observed between red deer and wapiti (d=0.0248) was less than between European and North American moose or between reindeer and caribou (Dratch and Gyllensten 1985).

The phylogenetic relationship among Cervidae, although not specifically between red deer and wapiti, has been explored using karyotyping (Fontana and Rubini 1990), repetitive DNA (Lima-de Faria *et al.* 1984, Bogenberger *et al.* 1987, Scherthan *et al.* 1990), RAPD analysis (Comincini *et al.* 1996), RFLP analysis of total mtDNA (Cronin 1992), and RNA gene sequences of mtDNA (Miyamoto *et al.* 1990). Karyotyping has shown admixtures in populations where sika and red deer hybridize (Bartos and Zirovnicky 1982). However, Fontana and Rubini (1990) have shown chromosome numbers for red deer and wapiti range from $2n = 68$ to $2n = 66$ and sika deer can exhibit these, as well as chromosome numbers as low as $2n = 62$.

To infer the phylogenetic relationship among red deer, wapiti, and sika deer, a phylogeny was constructed using the control region of mitochondrial DNA (mtDNA). Other North American cervids, including moose, white-tailed deer (*Odocoileus virginianus*), black-tailed deer (*O. hemionus columbianus*), and caribou (*Rangifer tarandus*) were also included in the phylogenetic analysis.

Materials and Methods

Collection

Rocky Mountain wapiti, moose, and white-tailed deer samples were collected opportunistically from Banff National Park, AB. The Manitoban wapiti was supplied by Riding Mountain National Park, MB. Black-tailed deer and Roosevelt wapiti were supplied by the Alberta and British Columbia Fish and Wildlife Services. The Forensic Laboratory of the United States Fish and Wildlife supplied lyophilized samples from sika deer, red deer, and Tule wapiti. Cansibmaral Elk LTD supplied DNA samples for the Siberian wapiti (*C. e. sibericus*). The following Genbank accessions were included obtained: bison (*Bison bison*) Accession No. U12933 by Beech, Sheraton, Polziehn, and Strobeck (unpublished data); domestic sheep (*Ovis aries*) Accession No. L29055 by Zardoya *et al.* (1995); Chinese water deer (*Hydropotes inermis*), roe deer (*Capreolus capreolus*), and brocket deer (*Mazama* sp.) Accession Nos. Y08207-Y08209 and Z70318 by Douzery and Randi (1997); and another sika deer and red deer Accession Nos. U12867-U12868, respectively by Feng, Li, Rittenhouse, and Templeton (unpublished paper). The caribou sequences for the control region were obtained from research associate John Coffin and graduate student Greg Dueck.

Isolation and Amplification (PCR)

DNA was isolated by lysing blood cells as in Bork *et al.* (1991) or using methods described in the Qiagen QIAamp Tissue Isolation kit. The control region of mtDNA and partial flanking tRNA genes were enzymatically amplified as described in Polziehn *et al.* (1996). The amplified products were purified on 1 % agarose 0.5 X TBE gel and extracted from the agaorse using Qiagen QIAquick Extraction kit. Sequencing reactions of the control region were performed as described in the Perkin Elmer DNA Terminator Cycle Sequencing Ready Reaction kit on a 9600 Perkin Elmer Cetus thermocycler. Sequencing reactions were separated by electrophoresis on an ABI Prism 377 Perkin Elmer automated sequencer and analyzed using ABI software.

Phylogenetic Analysis

Sequences were aligned using software Sequence Editor™, and nucleotide substitutions, deletions, and insertions were identified. Sequences were analyzed for their phylogenetic content using the branch and bound option in the computer program PAUP 3.1 (phylogenetic analysis using parsimony, Swofford 1993). Trees were rooted using mtDNA control region sequences from bison, sheep, moose, caribou, white-tailed deer, black-tailed deer, Chinese water deer, roe deer, and brocket deer.

Estimate of Divergence

To measure the time of divergence, the number of substitutions per nucleotide (K) is estimated from the proportion of nucleotide differences (p) between two sequences: K=-3/4 *ln* (1-4/3p). The time of divergence (t) was calculated as: $t = K/2u$ where u is the rate of nucleotide substitution (Li and Graur 1991). The values for u for the control region are estimated at 0.118×10^{-6} substitutions/bp/year (Stoneking *et al.* 1992). Gaps greater than one nucleotide were weighted as a single event.

Results

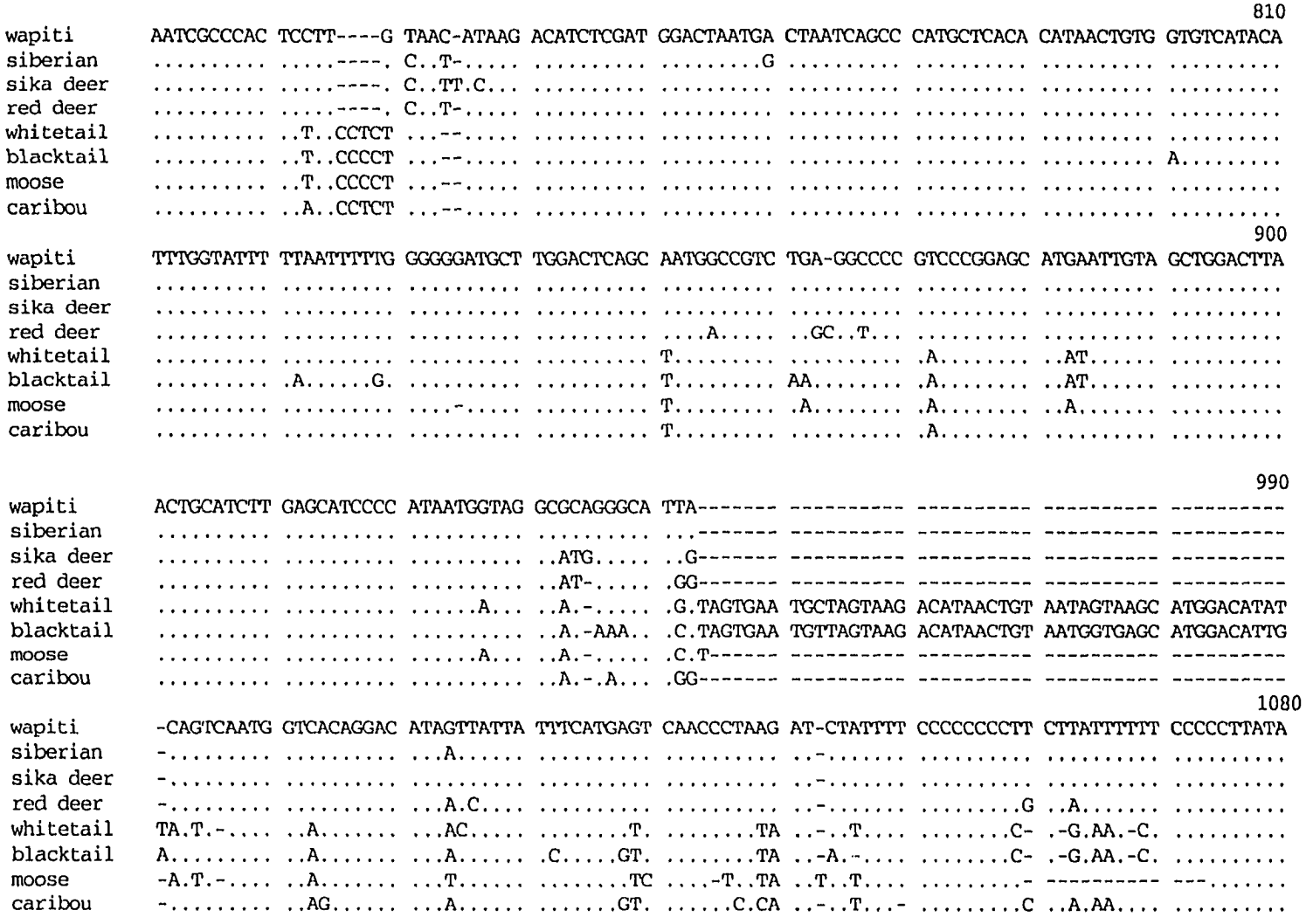
The amplified mtDNA from the control region, given in increasing size, was 1134 bp in red deer, 1145 bp in caribou, 1188 bp in moose, 1211 bp in wapiti, 1214 bp in sika deer, and 1267 bp in both white-tailed and black-tailed deer. Consensus sequences for wapiti, sika deer, and red deer are given in Table 1. Sequences submitted to Genbank were assigned the following Accession Nos. AF005196-AF005200, AF0058369-AF0058371, and AF016951-AF016980.

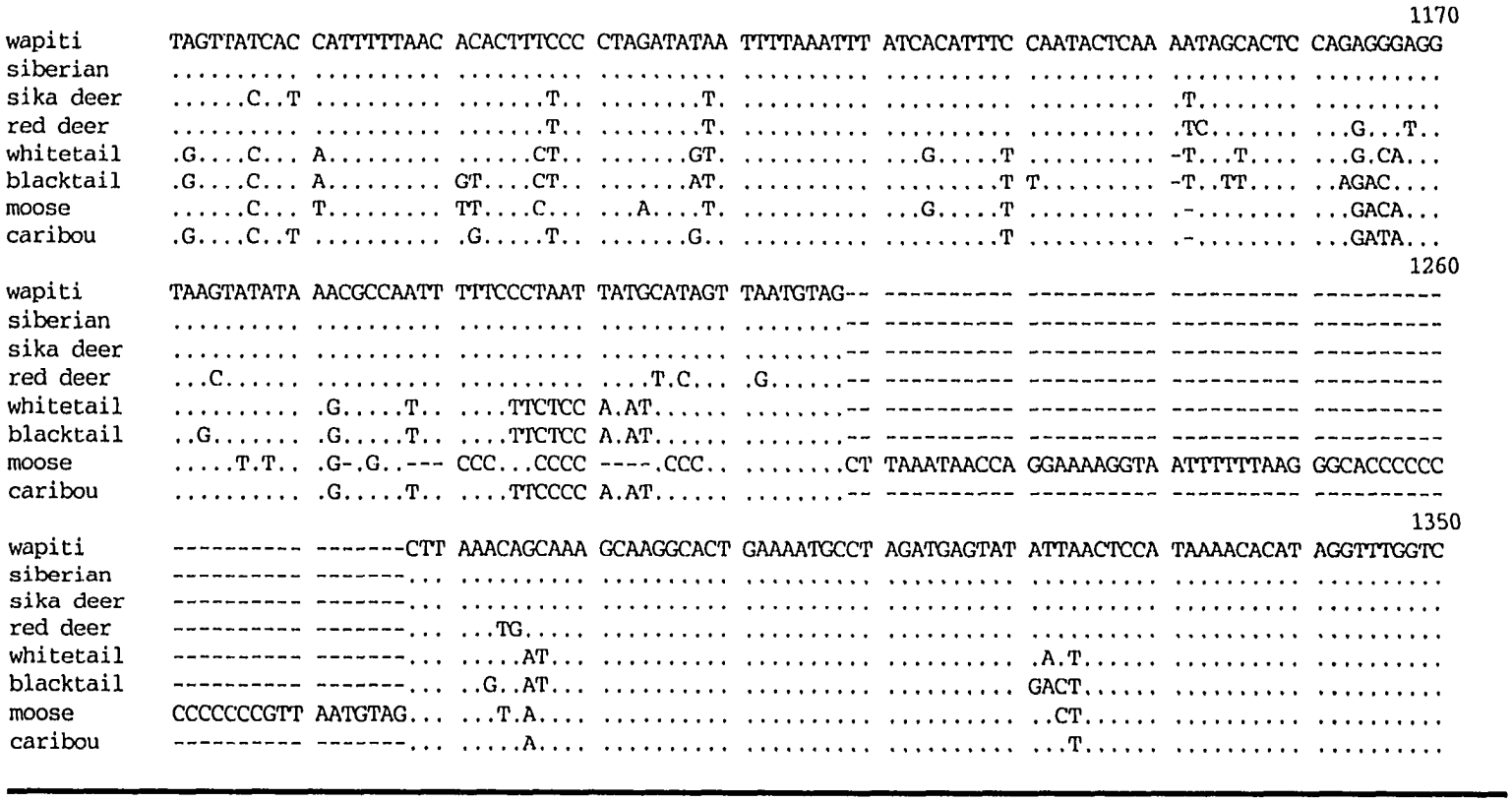
To determine the relationship among *Cervus elaphus*, cervid sequences were aligned to *Ovis aries* and optimized when possible for two character states per nucleotide site. A heuristic search in PAUP produced eight most parsimonious trees (CI = 0.815) requiring 1068 steps (Figure 4-2). The 100 bootstrap replicates showed greater than 50 % support for all nodes. A heuristic search including bison produced six parsimonious trees (CI = 0.705) of 1173 steps. Note the Chinese water deer (*Hydropotes*) moved from an expected sister group to Odocoileinae to join the brocket deer (Mazama) within this subfamily. All nodes occur with 100 % frequency except the polytomy within North American wapiti that was found in 83 % of the bootstrap replicates (Figure 4-3a). However, Saccone *et al.* (1991) recommended only the conserved region of the control region be used for phylogenetic construction because the right and left domains evolve in a species-specific manner. Therefore, the data was reanalyzed with positions 671-680,

Table 4-1. Sequences from the control region of mitochondrial DNA.

```
                                                                                                                    90
wapiti      TAATATACTG GTCTTGTAAA CCAGAAAAGG AGAGCAACCA ACCTCCCTAA GACTCAAGGA AGAAGCCATA GCCCCACTAT CAACACCCAA
siberian    .......... .......... .......... .......... .......... .......... .......... .......... ..........
sika deer   .......... .......... .......... .......... .......... ..-....... .......... .......... ..........
red deer    .......... .......... .......... ........T. .......... ..-....... .......... .......... ..........
whitetail   .......... .......... .......... ........T. .T........ .......... .......... .......... ..........
blacktail   .......... .......... .......... .......... .T......G. .....G.... .......T.. .......... ..........
moose       .......... .......... .......... ..TA...... .......... .......... ......T... .......... ..........
caribou     .......... .......... .......... ...-T..T.. .T........ .......... ......T... ..T....... ..........
                                                                                                                   180
wapiti      AGCTGAAGTT CTATTTAAAC TATTCCCTGA CGCTTATTAA TATAGTTCCA TAAAAATCAA GAACTTTATC AGTATTAAAT TTCCAAAAAA
siberian    .......... .......... .......... .......... .......... .......... .......... .......... ..........
sika deer   .......... .......... .......... .......... .......... .......... .......... .......... ..........
red deer    .......... .......... .......... T......... .......... .......... .......... .......... .........G
whitetail   .......... .......... .......... ...A...... .....C.... .....CC... ..G....... .......... ..TT-.....
blacktail   .......... .......... .......... ...A...... .....C.... C....TC... ..G.C..G.. .......... ...TT.....
moose       .......... .......... .........G ...A...... .......... C....T.... ....C..... .......... .CTT-.....
caribou     .......... ...A...... .........G ..TA...... .....C.... C....T.... ..G.C..G.. .......... ...T-.....
                                                                                                                   270
wapiti      --TTTAATAT TTTAATACAG CTTTCTACTC AACATCCAAT TTACATTTTA TGTCCTACTA --ATTACAC- AGCAAAACAC GTGATATAAC
siberian    --........ .......... .......... .......... .......... .......... .......... .......... A.........
sika deer   -T........ .......... .......... ....C..... .......C.. ..C....T.. .......... .A....G... ..........
red deer    -T........ ..C....... .....C.... ....C...T. .......... CA...-..C. ...CC..... .A.....T.T ..A...A...
whitetail   -T.......A .......... T...G..... ..T.G...TA .......C.T .A-A-...C. ..-....CTA CA....C--T .CA---C...
blacktail   -T..C....A .......... T...GC...T ....G...TA .........T CA-A-C.... ..-C...CTA CA....C--T .CATA.....
moose       TC.....C.A .......... T...G..... ....G...TA ...-..A..T CA.A-...C. ..-...ATTA CA.T..G--T ACATA..G.T
caribou     CC..C..G.A .......... T.C.GC.... ..T.G...TA ...T..CC.. AA.AG..-.. TC....ACTA CA-T...--T A.TT.....-
                                                                                                                   360
wapiti      ---------- ---------- --CTTATGCG CTCGTAGTAC ATAAAATCAA TGTGCTAGGA CATGC-ATGT GTAACAGTAC ATGA-GTTAG
siberian    ---------- ---------- --........ .......... .......... .......... ...A.-.... A......... ....-.....
sika deer   ---------- ---------- --......T. ..T....... .......T.. ..CATC.A.. ...A.T.... ACGG...... ....AACCG.
red deer    ---------- ---------- --........ ..TA...... ...G...T-- ---------- ---------- ---------- ----------
whitetail   --A-ATGTAT TTATTATATA ATT....... GGT....... .......T.. ...ATC.A.. ...ATT.... A...T..... ..T.CA..--
blacktail   --ACATGCAT -----ATATA ACT....A.. GGTA...... .......T.. ...A...... ...ATT.... A...T..... ..A.CA..--
moose       TTATATGCAT -CAGTAC... -T-C...A.. GGTA...... ..-------- ---------- ---------- ---------- ----------
caribou     --AC--G-GT CCTGTA---- --------.. G-TA------ ---------- ---------- ---------- ---------- ----------
```

```
                                                                                                                    450
wapiti      CGTATAGGAC ATATTATGTA TAATAGTACA T-AAATTAAT GTATTAAGAC ATATTATGTA TA-------- --ATAGTACA TTATATTATA
siberian    .......... .......... .......... .-........ ......G... .......... . -------- --........ ..........
sika deer   TA........ ...C...... .......... .-........ ......GA.. .......... ..-------- --........ ..........
red deer    ---------- ---------- ---------- -------... ...C..G... ...C...... ..-------- --........ ..........
whitetail   -A....CCC. ...C.-.A.. ACGA...... .A........ .......A.. .......... ..-------- --........ ...A.C....
blacktail   -A....CCC. ..GC.-.A.. AGCG...... .A........ .......... .......... ..-------- --........ .C.C......
moose       ---------- ---------- ---------- -A.C...... ....C..... .......... ..-------- --........ ...C.C...C
caribou     ---------- ---------- ----...... .A........ ...C..G... .......... ..TACATATA TG........ ...A......
                                                                                                                    540
wapiti      TGCCCCATGC TTATAAGCAT GTACTTCTCA CTATCTGAAG TACATAGTAC ATAATGTTGT TCATCGTACA TAGTACATTA AGTCAAATCA
siberian    .......... .......... ......T... ......A... .......... .......... C......... . ........ ..........
sika deer   .......... .......... ...T..TCT. T.....AT.. .......... ..G....CA. .T........ ...C...... ..........
red deer    .......... A......... ......TCT. T...T.AT.. .......... ..G....... .......... ....G..... ..........
whitetail   CA........ ...C... .A ....A.ACA. TC.CT.C... .......... ...C.A..A. .A.....C.. .......... .........T
blacktail   .A........ .........A ....A.ACA. .C..T..C.. .......... ...CA...A. .A.....C.. .......... .........C
moose       .A........ .........A ....A.GA.. T...TA.T.. .......... ...T.A..A. .G........ .......... T........C
caribou     .......... .........A ......GA.. ....T.AC.. .....G.... .....A..A. .G........ .........G .........C.
                                                                                                                    630
wapiti      GTCCTTGTCA ACATGCGTAT CCCGTCCCCT AGATCACGAG CTTAATTACC ATGCCGCGTG AAACCAGCAA CCCGCTGGGC AGGGATCCCT
siberian    .......... .......... .......... .......... .......... .......... .......... .......... ..........
sika deer   .......... .......... .......... .......... ...G...... .......... .......... ......A... ..........
red deer    .......... .......... .......... .......... ...G...... .......... .......... .......... ..........
whitetail   AC...C.... .......... ........T. .......... .....CC... .......... ......T... ......T... ..........
blacktail   C.T....C.. .......... .......... .......... .......... .......... ......A... ......T... ..........
moose       A.T....... .......... .......AT. .......... ......C... .......... ......A... ......T... .A........
caribou     T.T....... .......... .......... .......... .......... .......... .......... ......T... ..........
                                                                                                                    720
wapiti      CTTCTCGCTC CGGGCCCATG AACCGTGGGG GTAGCTATTT AATGAATTTT ATCAGACATC TGGTTCTTTT TTCAGGGCCA TCTCATCTAA
siberian    .......... .......... .......... .......... ...A..C... .......... .......... .......... .....C....
sika deer   .......... .......... .......... ...A...... ......C... .......... .......... .......... .....C....
red deer    .......... .......... ...T...... .......... ......C... .......... .......... .......... .....C....
whitetail   .......... .........A T.AT...... .......... ......C... .......... .........C .......... .....C....
blacktail   .......... .........A T.TT...... .......... ......C... .......... .........C .......... .....C....
moose       .......... .......... C..T...... .........C ......C... .......... .........C .......... .....C....
caribou     .......... .......... T.T....... .......... ......C... .......... .........C .......... .....C....
```





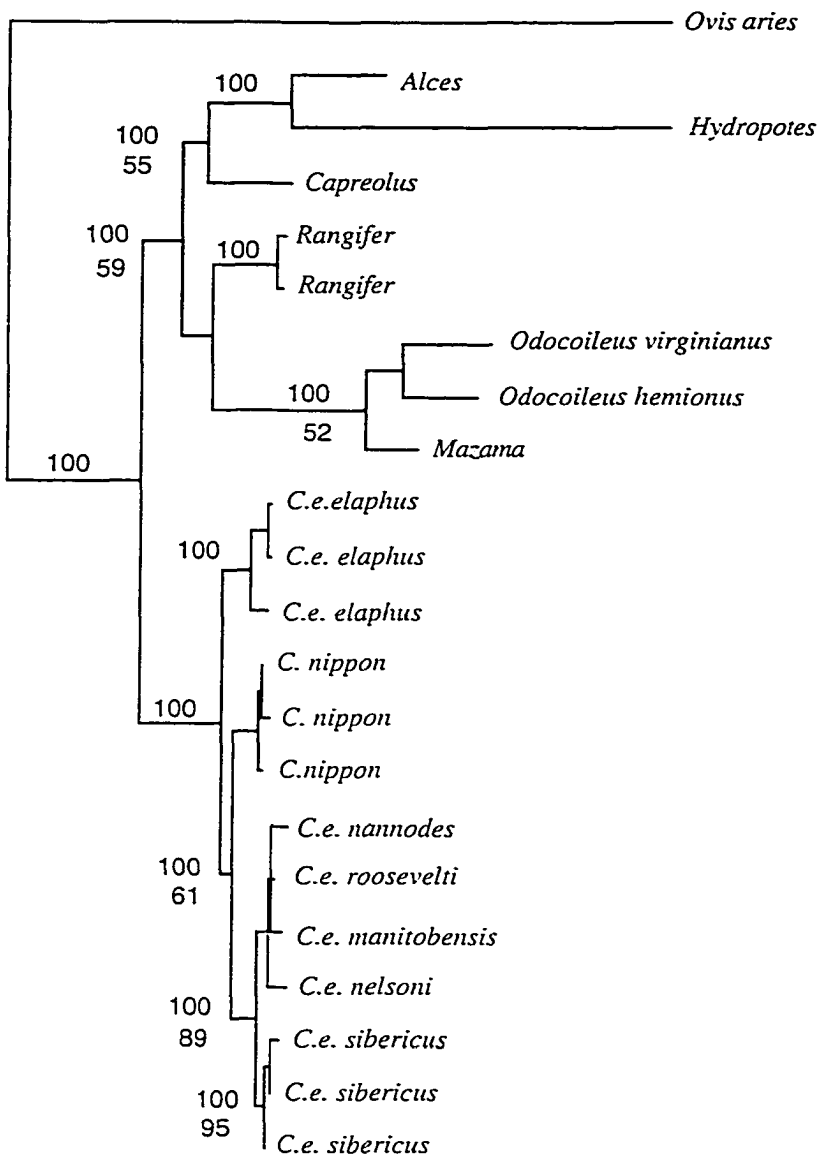


Figure 4-2. The phylogenetic relationship among wapiti, red deer, sika deer, roe deer, and other North American cervids using sequence data from the control region of mtDNA. The majority rule consensus tree of 8 parsimonius trees with a CI = 0.815 and 1068 steps. A representative phylogram is depicted. Bootstrap support for each node is noted below the frequency observed in the consensus tree. Bootstrap values less than 50 % are not shown.

783-1010, and 1052-1202 removed from the left domain (references alignment to the *Ovis* sequence in Zardoya *et al.* (1995)). A heuristic search in PAUP produced four parsimonious trees (CI = 0.656) requiring 665 steps, and showed the Siberian wapiti belong to within the North American clade (Figure 4-3b). Bootstrap replicates showed 100

% support for all nodes. The consensus tree (Figure 4-2) was also produced when additional non-conserved regions were removed from the right domain (data set excluding bison). These eight most parsimonious trees (CI = 0.738) used only 753 steps.

The Cervinae clade was monophyletic using all data sets, however the Odocoileinae clade was paraphyletic when the total sequence was employed. Discrepancies between the complete and conserved data sets occurs in the alignment of the right and left domains of the control region, which are prone to insertions, deletions and slippage. Heterogeneity in the right and left domains however, may provide valuable information for determining the relationship among less diverged species.

Members of the subfamily Odocoileinae listed in Table 4-1 have 30 autapomorphic nucleotide substitutions and a 22 bp insert in the left domain that sets it apart from the subfamily Cervinae. All trees constructed from the conserved regions of the control region support a distinction between the two subfamilies. *Alces alces* (moose) and *Rangifer* (caribou) were lacking the 75 - 77 bp repeat found in other species. *Alces alces* also has a large 59 bp insert and a 14 bp deletion in the right domain, while *Rangifer* has a short 10 bp insert in the left domain of the control region. A portion of the large insert was also found in the bison sequence. *Alces alces* and *Rangifer* were found to vary by 11.1%, which corresponds to 470,339 years of divergence between the two species.

The *Mazama* species (brocket deer) and *Odocoileus* species (black-tailed and white-tailed deer) shared a large 48 bp insert in the right domain which suggests a common ancestry between these two species. Comparisons of white-tailed deer and black tailed deer revealed 69 transitions, 21 transversions, and six deletions/ insertions, corresponding to 7.58 % divergence between the two species. Estimates of the time of divergence between the two species was estimated at 351,353 years ago.

Sequences from the control region of mtDNA of *Cervus elaphus* subspecies revealed nearly an equal amount of substitutions between sika deer, red deer, and wapiti. Red deer and sika deer varied by 49 transitions, 8 transversions, 1 insertions, and 3 deletions. Red deer and wapiti varied by 52 transitions, 11 transversions, 2 insertions, and 4 deletions. Wapiti and sika deer varied by 54 transitions, 3 transversions, 4 insertions, and 1 deletions. The Siberian wapiti varied from red deer by 46 transitions, 11 transversions, 2 insertions and 4 deletions. The Siberian wapiti varied from sika deer by 51 transitions, 2 transversions, 4 insertions, and 1 deletion. The one deletion observed in red deer is the 76 bp repeat found in both wapiti and red deer. Similar repeats have been found in many species (Douzery and Randi 1997), with four copies observed in *Ovis aries* (Zardoya *et al.* 1995). The repeat was found in population of moose in Sweden but

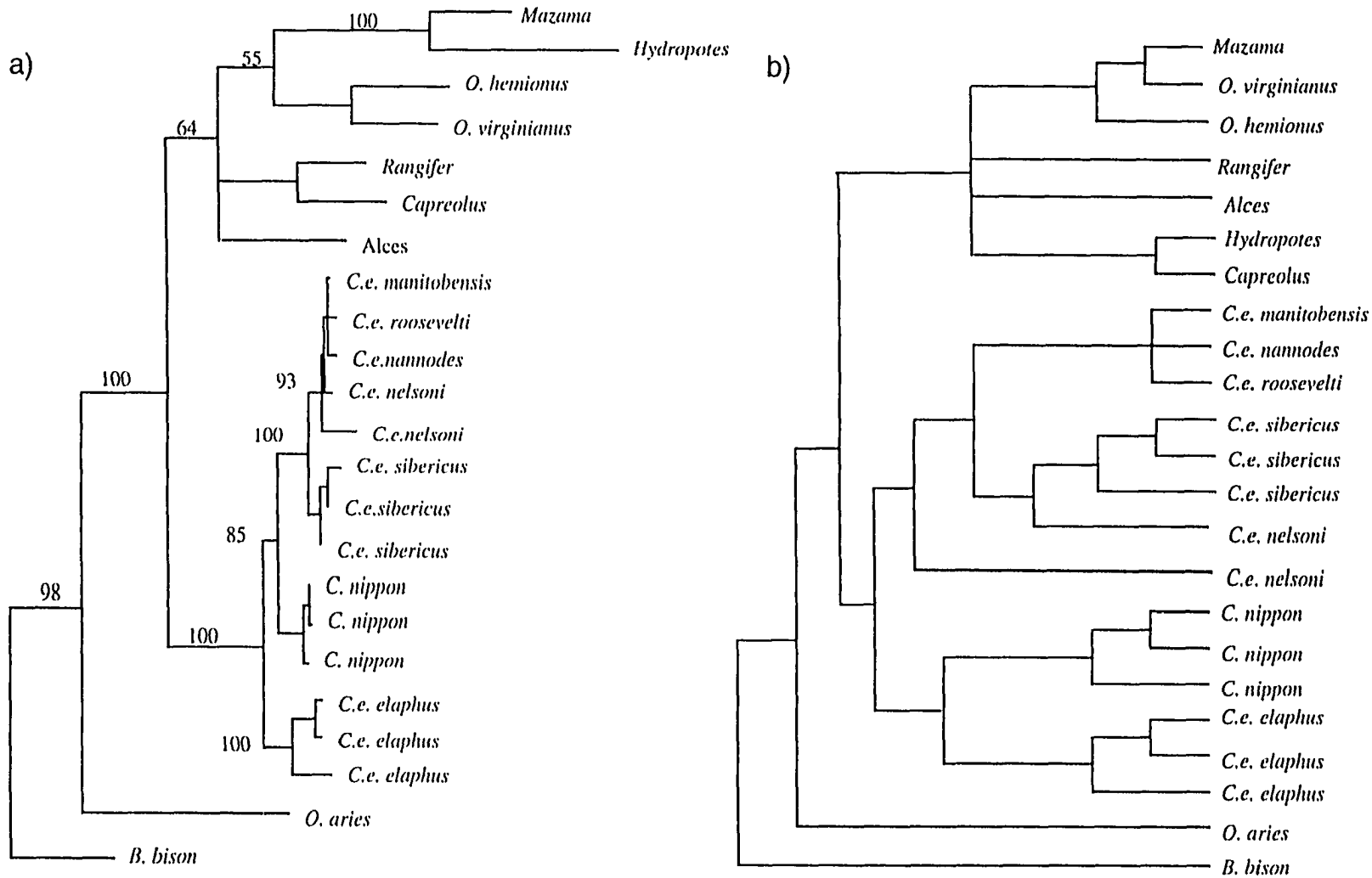


Figure 4-3. The phylogenetic relationships among the Cervinae in these two trees are based on varying amounts of conserved sequences in the control region of mtDNA. The CI are low for both trees; however, they indicate possible relationships that should not be overlooked. (a) A concensus of six most parsimonius trees requiring 1173 stpes with a CI = 0.705. Bootstrap values greater than 50 % are marked at each node. Note the position of *Hydropotes*. (b) A concensus tree of four most parsimonious trees with a CI = 0.656 and requiring 665 steps. Nodes are produces with 100 % frequency.

not North America (Mikko and Andersson 1995). Little phylogenetic information may be gained from repeats because they may vary in number and within species. If the 76 bp deletion in red deer was counted as a single mutation, the divergence between sequences was 5.60 % between wapiti and red deer, 5.19 % between wapiti and sika deer, and 5.02 % between red deer and sika deer. The average sequence divergence translates into an estimated 247,000 years since red deer and wapiti diverged, 228,000 years since red deer and sika deer diverged, and 220,000 years since sika and wapiti diverged. The shift in the position of wapiti as a sister group to red deer in Figure 4-3 to a sister group to both sika and red deer suggests a tenuous relationship among these three species.

In the comparison of Siberian and North American wapiti mitochondrial sequences for the control region, a range of 6 - 21 nucleotide substitutions was observed. The greatest number of differences was found between Siberian and Tule wapiti, with the least number between Rocky Mountain and Manitoban. A similar range of 0 - 15 nucleotide substitutions was observed among North American wapiti, where the greatest distance was between Tule and Roosevelt animals, and the least distance between Rocky Mountain and Manitoban animals (Polziehn and Strobeck 1998). Siberian wapiti could be distinguished from the North American wapiti by three unique substitutions at sites 261, 511, and 674. Twelve additional variable sites in Siberian wapiti were also observed to vary in North American wapiti, sika deer, and/or red deer. Siberian wapiti usually grouped outside the North American animals, however, the strong similarity of Siberian wapiti and Rocky Mountain wapiti is shown in Figure 4-3 where North American and Siberian wapiti appear to share a recent common ancestor. The estimated time of divergence for Siberian and North American wapiti, which vary by 1.23% in their sequences, is 52,000 years.

Conclusion

Recognition of the taxonomic status of a species plays a significant part in the management, protection, and understanding of the species. Recently, comparisons between molecular characters have been employed to complement the traditional morphological and ecological characteristics that define taxa. Since researchers began using protein electrophoresis (Bonnel and Selander 1973, Cameron and Vyse 1976, Ryman *et al.* 1977) they have found little variation among large mammals, and in more recent years, the focus has moved to comparisons of DNA. Genetic markers have been used to separate order Artiodactyla into suborders (Modi *et al.* 1996), suborder Ruminantia into superfamilies Bovoidea and Cervoidea (Comincini *et al.* 1996), and

family Cervidae into subfamilies (Lima de Faria *et al.* 1984; Miyamoto *et al.* 1990; Comincini *et al.* 1996; Cronin *et al.* 1996).

The diversity of cervid fossils from the Pleistocene, including ancestors of present day animals and extinct cervids, such as *Cervalces* and *Megaloceros giganticus* (Irish elk), suggests a wide radiation occurred during this time. Fossil records place Cervinae in the early Pliocene and Odocoileinae appeared in the middle Pliocene epoch. The separation between Cervinae and Odocoileinae is supported by sequence data of the mtDNA, UPGMA analysis of RAPD's by Comincini *et al.* (1995), and DNA sequence data from the K-Casein gene (Cronin *et al.* 1996). The control region of mtDNA also supports a strong separation between these two subfamilies, although the relationship of species within each rank has uncertainties.

Within the subfamily Odocoileinae, *Mazama* grouped with *Odocoileus*, which confirms the relationship observed by Douzery and Randi (1997). In one analysis (Figure 4-3), *Hydropotes* (subfamily Hydropotinae) grouped with the *Mazama*. Bouvrain *et al.* (1989) suggested the telemetacarpal condition and large medial opening of the temporal cannal support the placement of *Hydropotes* in the Odocoileinae subfamily. A close relationship between the brocket and Chinese water deer is not far-fetched when one considers the morphological similarities between these two groups. Both species are of similar size, females have an unusual number of four mammae, the presence of tusk-like canines, and an ability to swim great distances (Nowak 1991). Antlers that are absent in the Chinese water deer are only simple spikes in the brocket deer. The Chinese water deer is also known to hump its back when disturbed, a behavior that mimics the arched back of the brocket deer. Although *Hydropotes* and *Mazama* share many primitive features, in this study *Hydropotes* grouped with *Capreolus* in the tree constructions that used either the entire sequence or that had removed the largest variable sequences of the control region. This supports the relationship between *Hydroptes* and *Capreolus* observed by Miyamoto *et al.* (1990) and Douzery and Randi (1997). A genetic analysis that includes other species within the Cervidae will likely produce revisions to the present classification of the Hydropotinae.

While sequence divergence clearly separated Odocoileinae from Cervinae, the relationship among species within the cervid subfamilies remains uncertain. Moose and caribou most likely have descended from lineages that did not give rise to the *Odocoileus* or *Mazama* species. Lima de Faria *et al.* (1984) found *Alces* and *Rangifer* had more similarities between repeat sequences of DNA than *Rangifer* and other cervids, and perhaps sequence information from the *Pudu* (Pudu), *Hippocamelus* (Heumul), and *Ozotoceros* (Pampas deer) will shed some light on the relationship of these species.

Estimated times of divergence may not helpful for assessing the relationship among the Odocoileinae because sequences have diverged more than 10 %, which produces a large variation in the estimation. The saturation of transitional substitutions, presence of repeats, and ambiguity in sequence alignment in the right and left domains will also produce further uncertainty in estimates of divergence between species. However, sequence variability within the control region of mtDNA suggests that the distantly related cervids, such as roe deer and caribou, diverged over 450,000 years ago, while more closely related species such as white-tailed and black-tailed deer may have diverged as recently as 300,000 years ago. Divergence between Siberian and North American wapiti was estimated at 52,000 years ago, which corresponds to presence of the last land bridge between Alaska and Russia.

Integration of white-tailed deer (*O. virginianus*) into mule deer (*O. hemionus hemionus*) territory is widely documented. The black-tailed deer (*O. h. columbianus*), however, is biogeographically separated to a greater extent from the white-tailed deer by the Rocky, Cascade and Sierra Nevada Mountain chain, therefore producing a greater morphological and genetic distinction between the two groups. The 7.58 % divergence between white-tailed and black-tailed deer is in agreement with the 6-7 % mt DNA variation observed by Carr *et al.* (1986), Avise *et al.* (1987), and Cronin *et al.* (1988).

The distinction of North American wapiti as a species has been denied generally on the basis of morphology. However, the white lipped deer (*Przewalskium albirostris*) which is accepted to be quite different from deer of the genus *Cervus* shares remarkable similarity in its external appearance and antler mass with wapiti (Geist 1987). While cervids may appear phenotypically similar, Lowe and Gardiner (1989) and Schonewald (1994) found that skeletal measurements placed red deer and wapiti into separate clades. UPGMA analysis of protein data from 28 loci also separated red deer from wapiti (Dratch and Gyllensten 1985) and the RFLP analysis of complete mtDNA separated a Mongolian wapiti from the North American wapiti (Cronin 1992).

Emerson and Tate (1993) failed to distinguish the relationship among red deer, wapiti, and sika deer using protein analysis of 22 loci, however, they found that the genetic distance between red deer and wapiti (d = 0.32) was similar to that between sika deer and wapiti (d = 0.39). Their unrooted bootstrapped Fitch-Margoliash consensus tree split red deer from sika and wapiti with 85 % frequency, and a similar rooted Kitch tree alternatively split sika from red deer and wapiti with only 40 % frequency. Sequence data from the control region found sequence divergence between sika and wapiti to be 5.02 % and between red deer and wapiti to be 5.60 %. The parsimonious trees in this study split red deer from wapiti and sika deer with 100 % frequency. In contrast, Emerson and Tate's

(1993) most parsimonious bootstrap tree split sika deer from red deer and wapiti with only 51 % frequency. Analysis of the K-casein gene (Cronin *et al.* 1996) also was unable to discriminate among red deer, sika deer, and wapiti, although once again sika deer formed a sister group more often with wapiti.

Radiation of wapiti, red deer, and sika deer appears to have taken place long before the last land bridge disappeared between Siberia and Alaska, placing the origin of these species in eastern Asia. The similarity of the Siberian wapiti to the North America wapiti suggests a close relationship among Siberian and North American wapiti, and that the separation of red deer and wapiti has taken place west of the Altai Mountains. The exact division between wapiti and red deer will require further sampling of these cervids along the Tien Shan Mountains and the Gobi desert. Such a recent separation has not led to a biological separation of the species and the interbreeding observed between red deer and wapiti can also be observed among other members of the *Cervus* genus. Barriers caused by climate change, disappearance of the land bridge, and habitat loss have prevented populations from mixing and contributed to the accumulation of unique differences among each cervid species. The nucleotide diversity observed in the control region of the mtDNA among red deer, sika deer, and wapiti adds to the behavioural, ecological, and morphological evidence that recognizes a difference between these three cervids. In light of the divergence observed in this study, the status of wapiti as a subspecies of red deer should be reconsidered.

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Chapter 5

A Phylogenetic Comparison of Red Deer and Wapiti Using Mitochondrial DNA[1]

Abstract

A phylogeny was constructed for red deer/wapiti (*Cervus elaphus*) subspecies using sequence data from the control region of mitochondrial DNA (mtDNA). The tree was rooted using *Cervus nippon* (sika deer), *Cervus albirostris* (Thorold's white-lipped deer), and several Odocoileinae species. All subspecies of *Cervus elaphus* were derived from a single recent common ancestor, which is consistent with current taxonomy that recognizes the group as monophyletic. Although the *Cervus elaphus* bifurcated into red deer (European) and wapiti (Asian/North American) lineages, a phenetic comparison revealed wapiti share more nucleotide similarities with sika deer (*Cervus nippon*). The division between mtDNA haplotypes of red deer and wapiti corresponds to subspecies found on either side of the Himalayan Mountains, which suggests *Cervus elaphus* may not have a clinal distribution. Phylogenetic evidence from the constructed from the cytochrome B sequences also identified a separation between red deer (*Cervus elaphus*) and wapiti (*Cervus canadensis*) lineages.

Introduction

There has been some difficulty assessing the conservation status of *Cervus elaphus*, commonly known as red deer or wapiti, because there has been considerable disagreement on whether red deer and wapiti should be regarded as two subspecies (Bryant and Maser, 1982) and whether *C. elaphus* has been divided into too many subspecies (Nowack, 1991). The holoarctic distribution of *C. elaphus* has contributed to the suggestion that European and North American subspecies represent forms found at extreme ends of a cline (Geist 1987) where subspecies are categorized into three contiguous groups (Figure 5-1, Groves and Grubb, 1987) that give the impression of a gradient in phenotypes. In contrast, Whitehead (1972) and Cockerill (1984) argue that a

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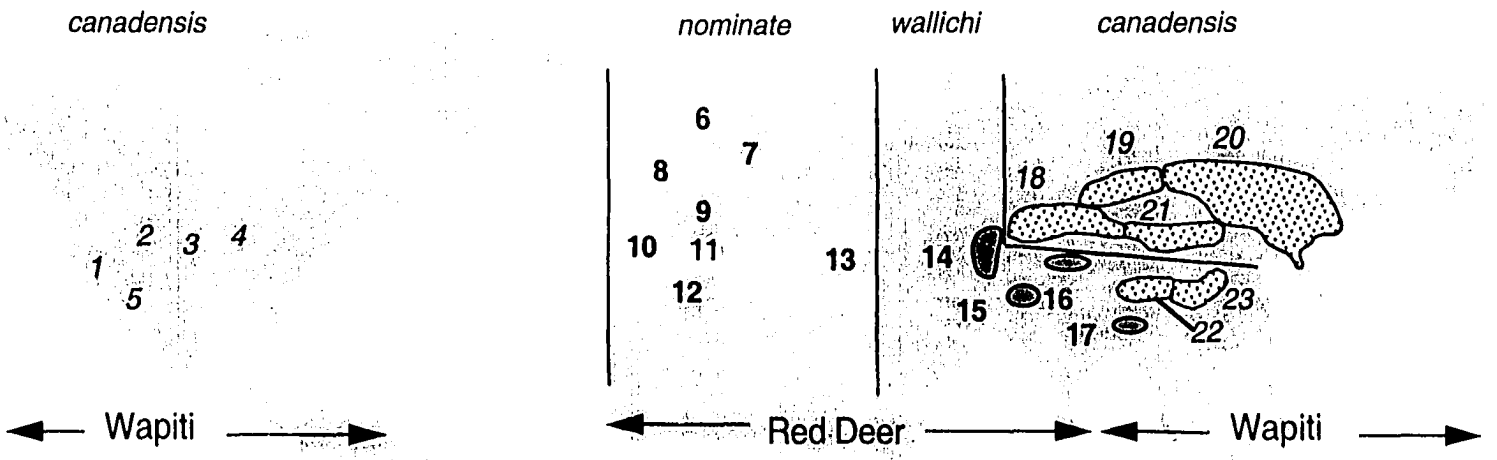

Figure 5-1. The distribution of *Cervus elaphus* subspecies as divided into three groups known as the nominate, wallichi, and canadensis by Groves and Grubb (1987) or into wapiti (italics) and red deer (bold) groups as described by White (1972) and Cockerill (1984). Mitochondrial haplotypes for red deer are shown in a solid color and wapiti in a pattern where their distinction may be dubious. Numbers that indicate the subspecies are respectively: 1 *roosevelti* (Roosevelt), 2 *nelsoni* (Rocky Mountain), 3 *manitobensis* (Manitoban), 4 *canadensis* (Eastern), 5 *nannodes* (Tule), 6 *atlanticus* (Norwegian), 7 *elaphus/typicus* (Swedish), 8 *scoticus* (Scotish), 9 *hippelaphus* (European), 10 *bolivari/hispanicus* (Spanish), 11 *corsicanus* (Corsican), 12 *barbarus* (Barbary), 13 *maral* (Maral), 14 *bactrianus* (Bactrian), 15 *hanglu* (Kashmir), 16 *yarkandensis* (Yarkand), 17 *affinis/wallichi* (Tibetan), 18 *songaricus* (Tien Shan), 19 *sibiricus* (Siberian/Altai), 20 *xanthopygus* (Manchurian), 21 *alashanicus* (Alashan), 22 *macneilli* (MacNeills) and 23 *kansuensis* (Kansu).

distinction between red deer and wapiti corresponds to the geographic barriers presented by the Gobi Desert and Himalayan Mountains and that the differences were sufficient to raise wapiti to *C. canadensis*. To assist the understanding of relationships among *C. elaphus* subspecies, their biogeography, morphology, and molecular relatedness are discussed below.

The current distribution of *C. elaphus* subspecies does not reflect the long periods of separation among Asian and European populations. *Cervus* species, thought to have originated from Asia, have appeared with coronate (crown-like) and acoronate forms in Europe during the 1-Gunz II, C-Cromerian, 2-Mindel, and D-Holosteinian interglacials (Dolan, 1988), which covers a time span from about 600,000 to 200,000 years ago. Glacial periods cycling about every 100,000 years ago (Webb and Bartlein, 1992) may have enforced long periods of separation between the deer populations and possibly provided the opportunity for European and Asian populations to differentiate. During the Riss or Salian glacial period, from about 200,000 - 150,000 years ago, most of northern Europe and western Siberia was covered with ice, central Europe was steppe Tundra, and southern Europe was dry steppe (Adams and Faure, 1997). Likewise, northern Asia was covered with desert, steppe tundra and ice while southern Asia was predominantly grassland (Adams and Faure, 1997). Connecting Europe and Asia was a bridge of semi-desert or desert that would have restricted any movement of deer between the two regions. Although a warming phase took place during the Eemian interglacial (130,000 - 115,000 years ago), it was not until the last 10,000 years that Europe and Asia were reconnected with vegetation that would have promoted movement of deer between the two regions (Adams and Faure, 1997).

Observations recorded throughout the 1600's to 1900's that discriminate red deer from wapiti (Bryant and Maser, 1982) are testimony to the presence of morphological and behavioral traits that separate the two groups. Neck manes found on both sexes of wapiti are present in only red deer males, the high pitched bugle of the wapiti is replaced by a low pitched roar in the red deer, and wapiti form larger herds with more open habitat than red deer (Cockerill, 1984; Groves and Grubb, 1987; Nowak, 1991). Red deer tend to be smaller, darker, and have antlers where the tines (branches) cluster at the terminal ends as compared to wapiti that have antlers where the tines are more evenly spaced (Lydekker, 1893; Harper, 1945; Dolan, 1988). Color, size, and minor differences in antler conformation, however, may not be considered reliable characters in systematic assessments (Geist, 1990) as indicated by the variance observed within North American subspecies (Soper, 1945; Green, 1956; Blood and Lovaas, 1966; McCullough, 1969; Hutton, 1972).

In addition to the traditional morphological and behavioral traits, molecular markers recently have been used to support taxonomic distinctions. Comparisons of allozymes and mitochondrial DNA (mtDNA) from European red deer and North American wapiti clearly indicate separation between the two groups (Dratch and Gyllensten, 1985; Dratch, 1986; Cronin, 1992; Polziehn *et al.*, 1998). Although few protein variants were found to discriminate red deer from wapiti, mtDNA sequences from the control region of red deer indicated the loss of a large repeat and about 5 % sequence divergence from wapiti (Polziehn *et al.*, 1998). Sampling from the ends of a cline however cannot address the possibility of a gradient in characters across the range of a species (Karl and Bowen, 1999) or the randomness at which molecular markers are lost from a population (Avise, 1998). Discrete distributions of the molecular characters would support the possibility of a species distinction if the distribution coincided with the ranges of wapiti or red deer. Currently there is no molecular information from most Asian subspecies to determine if a gradient of characters exists between Europe and North America.

To further complicate the understanding of subspecies of *C. elaphus*, the sika deer (*Cervus nippon*) was also proposed to be another point in the continuum of the red deer - wapiti cline (Geist, 1987). Early allozyme comparisons found few diagnostic markers between sika and red deer (Lowe and Gardiner, 1975; Linnell and Cross, 1991) and likewise Emerson and Tate (1993) were unable to resolve the relationship among wapiti, sika and red deer using an analysis of 22 proteins. More recent studies show a slight bias toward grouping sika deer and wapiti using sequence data mtDNA (Polziehn and Strobeck, 1998; Kuwayama and Ozawa, 2000) and an ability to detect hybridization between sika and red deer in Britain using mtDNA and microsatellite markers (Abernathy, 1994; Slate *et al.*, 1998). An inability to interbreed among sika deer, red deer, and wapiti would certainly have assisted in defining these species, however fertile hybrids are produced from crosses within the genus *Cervus* as well as among the genera within Cervinae (Winans, 1913; Lowe and Gardiner, 1975; Abernathy, 1994; Tate *et al.*, 1995; Long *et al.*, 1998).

In this paper, variation in the control region of mtDNA from *C. elaphus* subspecies was employed to evaluate the division between red deer and wapiti and to determine whether variation within subspecies warranted their taxonomic rank. The control region was chosen on the basis of its rapid rate of evolution and absence of recombination. Included in the phylogenetic analysis were *C. e. elaphus* from Europe; *C. e. barbarus* from Algeria; *C. e. bactrianus* from Uzbekistan; *C. e. alashanicus*, *C. e. kansuensis*, *C. e. macneilli*, and *C. e. xanthopygus* from China; *C. e. asiaticus* from

Russia, and *C. e. nannodes*, *C. e. nelsoni*, *C. e. manitobensis*, and *C. e. roosevelti* from North America. A phylogenetic analysis was also performed using cytochrome B regions taken from the literature that included several subspecies noted above as well as subspecies *C. e. yarkandensis* and *C. e. songaricus* from China. For the purpose of this paper, subspecies found in the southern Tien Shan Mountains and west of Himalayas will be referred to as red deer and subspecies found to the east will be referred to as wapiti.

Materials and Methods

Collection

Hair samples were supplied for Manchurian wapiti (*C. e. xanthopygus*) and Kansu red deer (*C. e. kansuensis*) by Yan Lu, Beijing Zoo and Jianjun Peng, Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Hair from what is believed to be an Alashan wapiti (*C. e. alashanicus*) was supplied by the Shuangyang No.1 State Deer Farm, Jilin, China. Hair samples were made available for two white-lipped deer (*C. albirostris*), five Barbary red deer (*C. e. barbarus*), two Bactrian red deer (*C. e. bactrianus*), and six MacNeill's deer (*C. e. macneilli*) by the San Diego Zoo, California. DNA was isolated from 10-40 hair follicles per individual using the protocol described in the Qiagen QIAamp Tissue Isolation kit. Steven Fain from the Forensic Laboratory of the United States Fish and Wildlife, Oregon supplied four tissue samples from Roosevelt wapiti (*C. e. roosevelti*) identified as Oregon individuals 6 - 9 in a previous study (Polziehn *et al.*, 1998).

Sequencing of DNA

The control region of the mtDNA and partial flanking tRNA genes was enzymatically amplified in 100 μl reactions for all samples using primers CST 2 5'TAATATACTGGTCTTGTAAACC3' and CST 39 5'GGGTCGGAAGGCTGGGAC CAAACC3' (Polziehn *et al.*, 1998). The amplified products were separated from unincorporated primers by electrophoresis on a 1% agarose 0.5 X TBE gel, excised, and purified using the Qiagen Qiaquick Extraction kit. Sequencing reactions used 8 μl template DNA, primers CST 2, CST 39, CST 25 5'TCATGGGCCGGAGCGAGAA GAGG 3', and CST 29 5'CGTGAAACCAGCAACCCGCTAGGC3'; and the Perkin-Elmer DNA Terminator Cycle Sequencing Ready Reaction mix. All amplifications were performed on a 9600 Perkin-Elmer Cetus thermocycler. Sequencing products were separated by electrophoresis on a 48 cm long 4 % acrylamide gel and detected using a ABI 377 Prism Perkin-Elmer automated sequencer and analyzed using ABI software.

Sequences of the control region were obtained from Genbank for Siberian wapiti (*Cervus elaphus sibericus*), Rocky Mountain wapiti (*C. e. nelsoni*), Manitoban wapiti (*C. e. manitobensis*), Roosevelt wapiti (*C. e. roosevelti*), Tule wapiti (*C. e. nannodes*), red deer (*C. e. elaphus*), sika deer (*C. nippon*), moose (*Alces alces*), and white-tailed deer (*Odocoileus virginianus*) with accession numbers AF058371-AF058368, AFO16980, AF016977, AF016975, AF016972-AF0168, AF016963-AF016963, AF016955, and AF016952 (Polziehn *et al.*, 1998a; Polziehn *et al.*, 1998b); another red deer and sika deer with accession numbers U12867 - U12868 (Feng, Li, Rittenhouse, and Templeton; unpublished), caribou (*Rangifer tarandus*) with accession number AF096449 (Dueck and Strobeck; unpublished). Sequences from the cytochrome B (cyt b) region of mtDNA from *Cervus elaphus* subspecies *canadensis*, *xanthopygus*, *kansuensis*, and *scoticus* subspecies were taken from Kuwayama and Ozawa (2000, accession numbers AB021096 - AB021099); *alashanicus*, *kansuensis*, *songaricus*, *xanthopygus*, and *yarkandensis* were taken from Tamate *et al.* (unpublished, accession numbers AB019625-AB019630) and *elaphus* subspecies from Randi *et al.* (1998, accession number AJ000021).

Phylogenetic analysis

Sequences were aligned using Sequence Editor software and analyzed using the computer program Phylogenetic Analysis Using Parsimony 4.0b2a (PAUP; Swofford, 2000). A heuristic search option was used to construct trees for parsimony and maximum likelihood for the control region. An exhaustive search was employed for the shorter cytochrome B region. Gaps were analyzed either as uninformative or as new character states. To prevent the large repeats in the control region from biasing the results when gaps were included as new character states, the entire repeat sequence between 306 bp and 502 bp was removed. Trees were rooted for the control region using sequence data from the Odocoileinae subfamily (caribou, moose, and white-tailed deer) and the Cervinae subfamily (sika and white-lipped deer). Bootstraps were performed to a total of 100 replicates, and transversional mutations were weighted against transitional mutations to a maximum of 15:1.

Estimate of divergence

The time of divergence (t) was calculated as $t = K/2\mu$, where μ is the rate of nucleotide substitution and K is the proportion of nucleotide differences between two sequences (Li and Gruar, 1991). Inserts and gaps greater than one nucleotide were considered as a single event and the rate of nucleotide substitution was estimated at 0.0118

Table 5-1. Consensus sequences from the control region and partial flanking tRNA genes of mtDNA are listed for Thorold's white-lipped deer (*C. albirostris*), sika deer (*C. nippon*), European red deer (*C. e. elaphus*), Barbary red deer (*C. e. barbarus*), Bactrian red deer (*C. e. bactrianus*), Kansu wapiti (*C. e. kansuensis*), MacNeills wapiti (*C. e. macneilli*), Alashan wapiti (*C. e. alashanicus*), Siberian wapiti (*C. e. sibericus*), Manchurian wapiti (*C. e. xanthopygus*), and Oregon wapiti (*C. e. nelsoni*; RMt or *C. e. roosevelti*; Rvt). Nucleotides that are identical to the reference sequence are denoted by '.' and insertions or deletions by a '-'. The sequence underlined in the reference sequence is repeated again in one of the white-lipped sequences.

									80
Whitelipped	GAAAAGGAGA	ACAACCAACC	TCCCTAAGAC	TCAAGGAAGA	GGCCATAACC	CCACCATCAA	CACCCAAAGC	TGAAGTTCTA	
Sika deer	....	-.........	.........-	....	A......G..	....T.....	....	....	
Red deer 1	....	G.........	....	....	A......G..	....T.....	....	....	
Red deer 2	....	G....T....	....	....	A......G..	....T.....	....	....	
Barbary	....	G.........	....	....	A......G..	....T.....	....	....	
Bactrian	....	-.........	....	....	A......G..	....T.....	....	....	
Kansu	....	G...-.....	....	....	A......G..	....T.....	....	....	
MacNeills	....	G.........	....	....	A......G..	....T.....	....	....	
Alashan	....	....	....	....	A......G..	....T.....	....	....	
Siberian	....	G.........	....	....	A......G..	....T.....	....	....	
Manchurian	....	G.........	....	....	A......G..	....T.....	....	....	
Oregon	.......-...	G.........	....	....	A......G..	....T.....	....	....	

									160
Whitelipped	TTTAAACTAT	TCCCTGACGC	TTATTAATAT	AGTTCCATAA	AAACCGAGAA	CTTTATCAGT	ATTAAATCTC	CAAAAATTTT	
Sika deer	....	....	....	....	...T.A....	....	.......T..	......A...	
Red deer 1	....	....	....	....	...T.A....	....	.......T..	......G...	
Red deer 2	....	....	....	....	...T.A....	....	.......T..	......G...	
Barbary	....	....	....	....	...T.A....	....	.......T..	......G...	
Bactrian	....	....	....	.........G	...T.A....	....	.......T..	......A...	
Kansu	....	....	....	....	...T.A....	....	.......T..	......A-..	
MacNeills	....	....	....	....	...T.A....	....	.......T..	......A-..	
Alashan	....	....	....	....	...T.A....	....	.......T..	......A-..	
Siberian	....	....	....	....	...T.A....	....	.......T..	......A-..	
Manchurian	....	....	....	....	...T.A....	....	.......T..	......A-..	
Oregon	....	....	....	....	...T.A....	....	.......T..	......A-..	

```
                                                                                                                                240
Whitelipped     TAATATTTTA ATACAGCTTT CTACTCAACA CCCAATTTAC ATTTT-ATGT CCTACTAATT ACACAGCAAA ACACACGATA
Sika deer       .......... .......... .......... .......... ...C.-...C ....T..... .....A.... G...GT....
Red deer 1      ........C. .......... .C........ .......... .....-.CA. -.C..C..CC .T...A..G. .T.TGTA...
Red deer 2      ........C. .......... .C......T. .......... .....-.CAC -.C.....CC .T...A..G. .T.TGTA...
Barbary         ........C. .......... .C......T. T......... .....T.CAC T.C.....CC .T...A..G. .T.TGTA...
Bactrian        ........C. .......... .......... T......... ..C..-.CA. .AC.....CC ...T.A.... .....TA...
Kansu           .......... .......... .......... T......... .....-.C.. .......... .......... ....GT....
MacNeills       .......... .......... .......... T......... .....-.C.. .......... .......... ....GT....
Alashan         .......... .......... .......... T......... .....-.CA. .......... .......... ....GT....
Siberian        .......... .......... .......... T......... .....-.... .......... .......... .....T....
Manchurian      .......... .......... .......... T......... .....-.... .......... .......... .....T....
Oregon          .......... .......... .......... T......... .....-.... .......... .......... ....GT....

                                                                                                                                320
Whitelipped     TAAC-TTTAT GTACTTAC-G TACATAA-AT TAATGTACTG GGGCATACTA TGTACGGCAG TACATGAAA- CGATGTGTTA
Sika deer       ....C-C... ..G...GTA. .......A.. .....C.TCA A.A....... .......... .........C ..G.A.A.-.
Red deer 1      ....C-.... .C.....TA. ......GA.. .........A ..A....... ....TAAT.. .....----- ----------
Red deer 2      A...C..... .C.....TA. ......GA.. .........A ..A....T.. ....TAAT.. .....----- ----------
Barbary         A...C-C... .CG....TA. ......GA.. .......T.. ..A....T.. ....TAAT.. .....----- ----------
Bactrian        .-..C-C... .CG....TA. ......GA.. .......T.. ..A....... ....TAAT.. .....----- ----------
Kansu           ....C..... .CG...GTA. .......A.. C.....G..A ..A.G...-. ....GAA... .........GT TAGC..A.-.
MacNeills       ....C-.... .CG...GTA. .......A.. C.....G..A ..A.G.G.-. ....TAA... .........GT TAG...A.-.
Alashan         ....C-.... .CG...GTA. .......A.. C.....G..A ..A.G.G.-. ....TAA... .........GT TAG...A.-.
Siberian        ....C-.... .C...CGTA. .......A.. C.....G..A ..A.....-. ....TAA... .........GT TAGC..A.-.
Manchurian      ....C-.... GCG..CGTA. .......A.. C.....G..A ..A...G.-. ....TAA... .........GT TAGC..A.-.
Oregon          ....C-.... .CG..CGTA. .......A.. C.....G..A ..A...G.-. ....TAA... .........GT TAGC..A.-.
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                                                                                                                    400
Whitelipped     GGACATACCA TGTATAGTAG TACATAAACT TATGTATTAG GACATACTAT GTATAATAGT ACATAAATTA ATGTATTAGG
Sika deer       ........T. ......A... ........T. A......... A.....T... .......... ...------ ----------
Red deer        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Barbary         ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Bactrian        ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
Alashan         .......TT. ..C...A... ........T. A.......C. A......... .......... ...------ ----------
Kansu           .......TT. ......A... ........T. A......... .......... .......... ...------ ----------
MacNeills       .......TT. ......A... ........T. A......... .......... .......... ...------ ----------
Siberian        .......TT. ......A... ........T. A......... ......T... .......... ...------ ----------
Manchurian      .......TT. ..C...A... ........T. A........A .......... .......... ...------ ----------
Oregon          .......TT. ......A... ........T. A........A .......... .......... ...------ ----------
                                                                                                                    560
Whitelipped     ATATTATGTA TAATAGTACA TTATATTATA TGCCCCATGC TTATAAGCAT GTACTTCTTA TTATTTACAG TACATAGCAC
Sika deer       ---------- ---------- -......... .......... .......... ...T..TC.. ....C..T.. .......T..
Red deer 1      ---------- ---------- -......... .......... .......... ...T.CT... .......T.. .......T..
Red deer 2      ---------- ---------- -......... .......... .......... ...T.CTC.. .......T.. .......T..
Barbary         ---------- ---------- -......... .......... .......... .....CTC.. ...C...T.. .......T..
Bactrian        ---------- ---------- -......... .......... .......... ......TCC. ..G....... .......T..
Alashan         ---------- ---------- -......... .......... ...C...... ...T..T... C......A.. .......T..
Kansu           ---------- ---------- -......... .......... .......... ......T.C. .......A.. .......T..
MacNeills       ---------- ---------- -......... .......... .......... ......T.C. .......A.. .......T..
Siberian        ---------- ---------- -......... .......... .......... ......T.C. C...C..A.. .......T..
Manchurian      ---------- ---------- -......... .......... .......... .........C. CC.....A.. .......T..
Oregon Rvt      ---------- ---------- -......... .......... .......... ......T.C. C.....GA.. .......T..
Oregon RMt      ---------- ---------- -......... .......... .......... ......T.C. C......A.. .......T..
                                                                                                                    640
Whitelipped     ATGATGTTGT TCATCGTACA TAGCGCATTA AGTCAAATCA GTTCCTGTCA ACATGCGTAT CCCGTCCCCT AGATCACGAG
Sika deer       .......CA. .T........ ....A..... .......... ..C.T..... .......... .......... ..........
Red deer        .......... .......... ...TA..... .......... ..C.T..... .......... .......... ..........
Barbary         .......... .T.C...... .......... .......... ..C.T..... .......... .......... ..........
Bactrian        .......... .......... ...T...... .......... ....T..... .......... .......... ..........
Kansu           ........A. .T........ ....A..... .......... ..C.T..... .......... .......... ..........
MacNeills       ........A. .T........ .......... .......... ..C.T..... .......... .......... ..........
Alashan         ..A....... .......... ...T...... .......... ..C.T..... .......... .......... ..........
Siberian        ..A....... C......... ....A..... .......... ..C.T..... .......... .......... ..........
Manchurian      ..AG...... .......... ....A..... .......... ..C.TC.... .......... .......... ..........
Oregon Rvt      ..A....... .......... ...T.....G .......... ..C.T..... .......... .......... ..........
Oregon RMt      ..A....... .......... ...T...... .......... ..C.T..... .......... .......... ..........
```

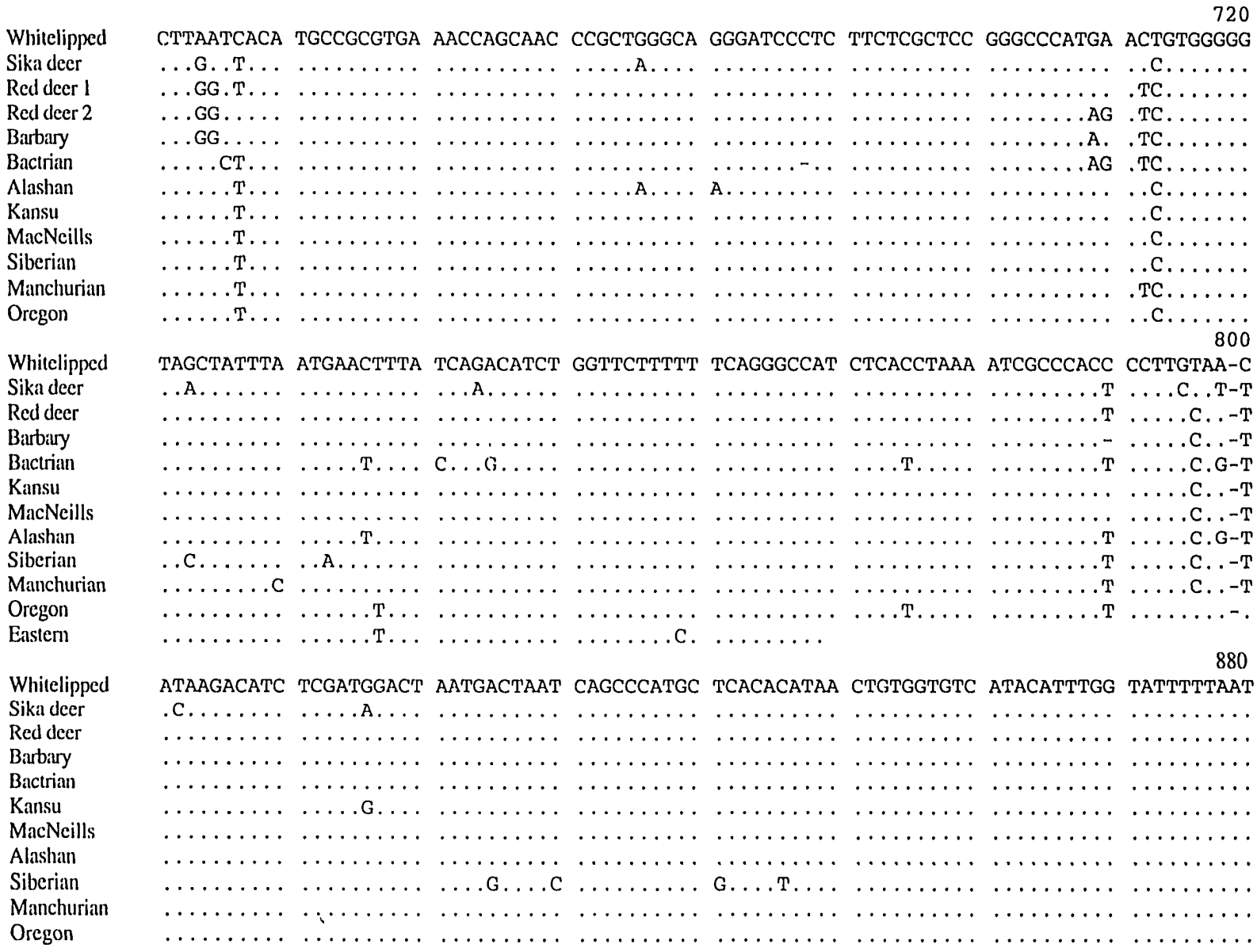

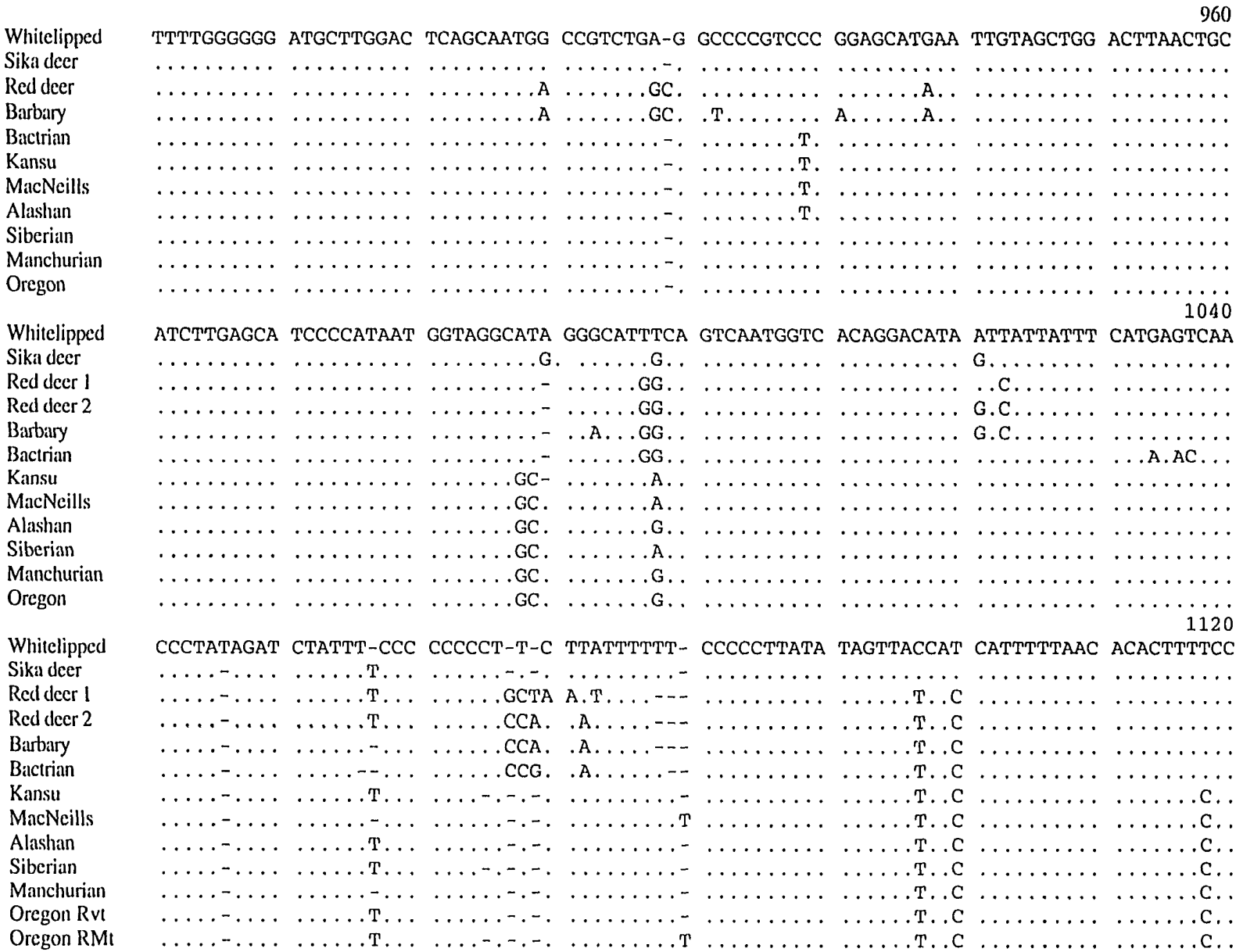

```
                                                                                                                1200
Whitelipped     CTAGATATTA TTTTAAATTT ATCACATTTT CAATACTCAA ATTAGCACTC CAGGGGGAGG TAAGTATATA AACGCCAATT
Sika deer       .......... .......... .........C .......... .......... .......... .......... ..........
Red deer 1      .......... .......... .........C .......... ..C....... .......T.. ...C...... ..........
Red deer 2      .......... .......... .........C .......... .......... .......T.. .......... ..........
Barbary         .......... .......... .........C .......... .......... .......T.. .......... ..........
Bactrian        .......... .......... .........C .......... .......... .......T.. .......... ..........
Kansu           ........A. .......... .........C .......... .A........ .......... .......... ..........
MacNeills       ........A. .......... .........C .......... .A........ .......... .......... ..........
Alashan         ........A. .......... .........C .......... .A........ .......... ..G....... ..........
Siberian        ........A. .......... .........C .......... .A........ .......... .......... ..........
Manchurian      ........A. .......... .........C .......... .A........ .......... .......... ..........
Oregon          ........A. .......... .........C .......... .A........ .......... .......... ..........
                                                                                                                1280
Whitelipped     TTTCTCTAAT TTTGCATAGT TAATGTAGCT TAAACGGCAA AGCAAGGCAC TGAAAATGCC TAGATGAGTA TATCAACTCC
Sika deer       ....C..... .A........ .......... .....A.... .......... .......... .......... ..........
Red deer 1      ....C..... .A....C... .G........ ....T.A... .......... .......... .......... ...T......
Red deer 2      ....C..... .A....C... .......... ....TAA... .......... .......... .......... .G.T......
Barbary         ....C..... .A....C... .......... ....TAA... .......... .......... .......... .G.T......
Bactrian        ....C..... .A........ .......... ....TA.... .......... .......... .......... ...T......
Kansu           ....C..... .A........ .......... ....CA.... .......... .......... .......... ...T......
MacNeills       ....C..... .A........ .......... ....CA.... .......... .......... .......... ...T......
Alashan         ....C..... .A........ .......... ....CA.... .......... .......... .......... ..........
Siberian        ....C..... .A........ .......... ....CA.... .......... .......... .......... ..........
Manchurian      ....C..... .A........ .......... ....CA.... .......... .......... .......... ...T......
Oregon          ....C..... .A........ .......... ....CA.... .......... .......... .......... ..........
```

x 10^{-6} substitutions/bp/year for the control region of mtDNA (Stoneking *et al*, 1992) and 0.10 x 10^{-6} substitutions/bp/year for the cytochrome B region of mtDNA (Irwin *et al.*, 1991).

Results

Sequences for the cervids were submitted to GenBank and assigned accession numbers AF296807-296823. The amplified products were 1131-1134 bp for the Bactrian deer (*C. e. bactrianus*), 1134-1135 bp for the Barbary deer (*C. e. barbarus*), 1209 bp for the Kansu wapiti (*C. e. kansuensis*), 1208-1210 bp for the MacNeills deer (*C. e. macneilli*), 1210 bp for the Alashan wapiti (*C. e. alashanicus*), 1211 bp for the Manchurian wapiti (*C. e. xanthopygus*), 1211-1212 bp for the Oregon wapiti (*C. e. roosevelti* and *C. e. nelsoni*), and 1291 -1330 bp for the white-lipped deer (*C. albirostris).*

The greater length variation in the sequences from Thorold's white-lipped deer was primarily caused by an increased number of repeats. As each repeat unit is 39 bp in length, the difference between the two white-lipped deer can be explained by one animal having seven repeats and the other having six repeats (Table 5-1). Wapiti and sika deer (*C. nippon*) in this study both have four repeat units while two repeats were observed in the red deer (*C. e. elaphus*) sequences. Because the number of repeat units in the control region of sika mtDNA can vary from four to six (Cook *et al.,* 1999), one may infer sika deer are more related to the white-lipped deer than either red deer or wapiti and that red deer have lost their repeats. Within the repeats, wapiti differ from the white-lipped and sika deer by five transitional changes and one indel. Although repeat units are common to the control region (Douzery and Randi, 1997), their phylogenetic usefulness has had limited evaluation (Lunt *et al.*, 1998; Cook *et al.,* 1999).

A slightly greater sequence similarity of the control region of mtDNA was also demonstrated between Thorold's white-lipped deer and sika deer when Thorold's white lipped deer was compared to sika, red deer, and wapiti. Sequence differences between Thorold's white-lipped deer and the most related sika deer, wapiti and red deer were 5.4%, 6.1%, and 6.3% respectively (Table 5-2), where 20 unique characters separated Thorold's white-lipped deer from other *Cervus* species.

When sequences from the control region of mtDNA were compared among the most related sika deer, red deer, or wapiti, sika deer and red deer had approximately 5.7 % differences between their sequences, sika deer and wapiti had approximately 4.5 % differences between their sequences, and red deer and wapiti had approximately 4.9 % differences between their sequences. Twenty-four unique characters separated wapiti and

red deer, including 15 transitional changes, 5 transversional changes, and 4 indels. An additional 13 unique transitional mutations and two indels distinguished the European and Barbary red deer from the Bactrian deer. Differences within the 11 sample groups listed in Table 5-2 were as high as 1.0 % difference among sika deer, 3.0 % among red deer and 0.8 % among wapiti sequences.

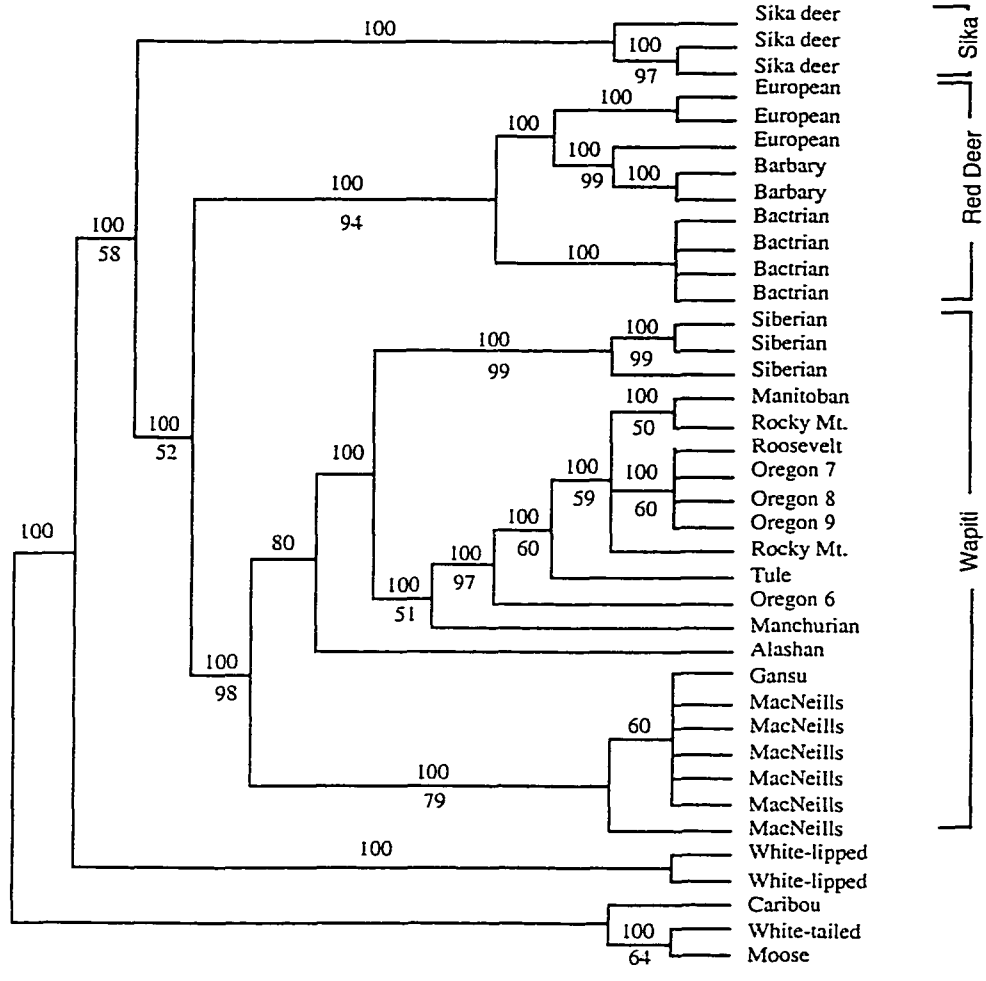

Figure 5-2. Phylogeny for *Cervus elaphus* constructed from the consensus of 15 equally parsimonious trees based on variation in the control region of mitochondrial DNA. The frequency of each node is noted above the line for the consensus tree and below the line for bootstrap values. Bootstrap values are not given if the frequency was below 50%.

To determine the phylogenetic relationship among *Cervus* species, sequences were aligned to caribou, white-tailed deer, and moose, and optimized where possible for no more than two character states per nucleotide site. In all analysis where characters were given equal weight, a clade was produced that contained a red deer lineage and a wapiti lineage.

Table 5-2a. Pairwise distances between sequences where values below the diagonal are the number of differences between the sequences and values above the diagonal are the mean character differences for the control region. American wapiti refers to Rocky Mountain, Manitoban, Roosevelt and Tule wapiti.

Name	Subspecies	Thorold	Sika	European	Barbary	Bactrian	American	Siberian	Manchur	Alashan	Gansu	McNeill
Thorold's	*prezwalski*	1	0.05354	0.06296	0.08063	0.07143	0.06137	0.05796	0.06137	0.06309	0.06061	0.05709
Sika	*nippon*	62-65	0-12	0.05730	0.06302	0.06487	0.04922	0.04659	0.05263	0.05086	0.04577	0.04494
European	*elaphus*	73-87	62-70	4-33	0.01758	0.0436	0.05741	0.05458	0.05556	0.05545	0.0519	0.04912
Barbary	*barbarus*	87-88	68-70	19-34	0	0.05009	0.06778	0.06679	0.06673	0.06673	0.06221	0.06060
Bactrian	*bactrianus*	77-78	70-71	47-57	54-55	0	0.05566	0.05571	0.05566	0.0538	0.05297	0.05200
American	*canadensis*	71-76	57-65	61-75	73-80	60-65	1-9	0.01121	0.01381	0.02155	0.01898	0.01898
Siberian	*sibiricus*	67-73	54-61	59-75	72-78	60-65	13-23	0-5	0.01293	0.02067	0.01638	0.01641
Manchurian	*xanthopygus*	71-72	61-65	60-70	72-73	60	16-21	15-20	0	0.02498	0.01726	0.18980
Alashan	*alashanicus*	73-74	59-64	60-71	72-73	58	25-30	24-29	29	0	0.02155	0.01641
Gansu	*kansuensis*	70-71	53-56	56-66	67-68	57	22-27	19-24	20	25	0	0.00346
McNeill's	*macneilli*	66-67	52-56	53-56	65-66	56-57	22-27	19-26	22-24	19-21	4-6	0-2

Table 5-2b. Pairwise distances between sequences where values below diagonal are number of differences between the sequences and values above the diagonal are the mean character differences for the cytochrome B region. American wapiti refers to Rocky Mountain, Manitoban, Roosevelt and Tule wapiti.

Name	*Subspecies*	European	Yarkand	TienShan	American	Manchurian	Alashan	Gansu
European	*elaphus*	7	0.03542	0.05450	0.05722	0.06540	0.06267	0.05995
Yarkand	*yarkandensis*	13	0	0.05009	0.04360	0.04087	0.04632	0.04087
Tien Shan	*songaricus*	20	15	4	0.00817	0.01907	0.01635	0.01362
American	*canadensis*	21	16	1	0	0.01635	0.01907	0.01362
Manchurian	*xanthopygus*	25	18	7	6	0	0.00272	0.01907
Alashan	*alashanicus*	23	17	6	7	1	0	0.01907
Gansu	*kansuensis*	23	15	5	5	7	7	1

A heuristic search produced 15 equally parsimonious trees requiring 574 steps (CI=0.714), where most branches in the consensus tree occurred with a 100 % frequency (Figure 5-2). Bootstrap for 100 replicates strongly supported several nodes within the red deer and wapiti lineages, but no among sika deer, red deer and wapiti.

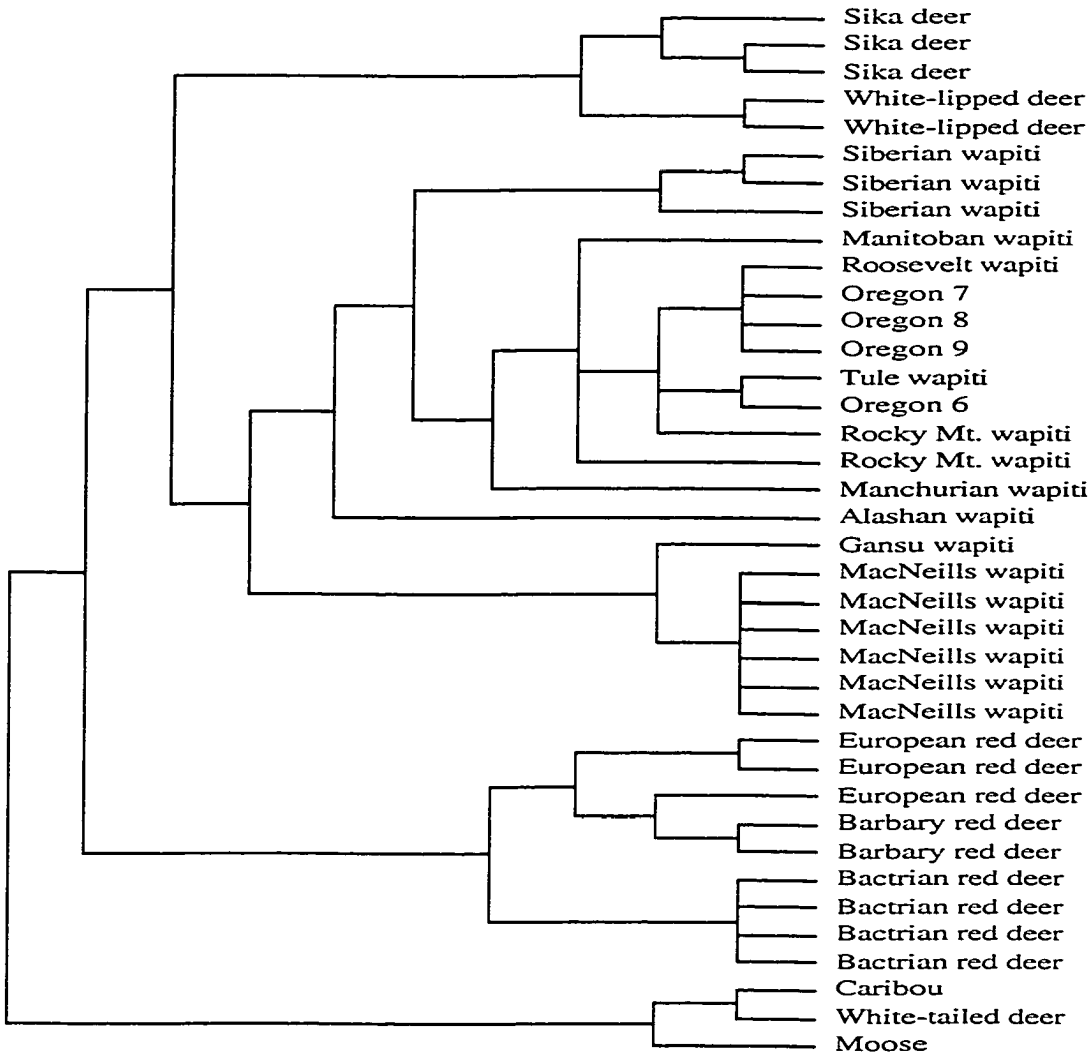

Figure 5-3. Phylogeny for *Cervus elaphus* constructed using Maximum Likelihood for sequences from the control region of mitochondrial DNA (-ln log = 4942.5631) when transversion to transition ratio 5:1.

Maximum likelihood based on Kimura two parameter was also used to determine the relationship among *Cervus elaphus* subspecies. A heuristic search using equal weighting of transitions to transversions produced an identical tree to the parsimony method (Figure 5-2). An increase in the weight from 1:1 of transversional to transitional mutations to 5:1 dramatically altered the topology of the maximum likelihood trees (Figure

5-3), however, increasing the weighting for transversions to a maximum of 15:1 did not continue to change the tree. When transversions were weighted, red deer were replaced by sika and white-lipped deer as the sister taxa to the wapiti group. The number of transversional mutations was nearly equal among sika, wapiti, red deer, and white-lipped deer, which suggests a similar divergence among all *Cervus* species and perhaps a need to reclassify subspecies of *C. elaphus*.

Distance analysis using neighbor joining trees based on Tajima and Nei with an uncorrected 'p' and assuming all sites are equally likely to change was used to produce a consensus tree from 24 trees with nearly the same topology as the consensus tree produced using parsimony method (Figure 5-2). However, the Alashan wapiti grouped with the Kansu and MacNeills wapiti (trees not shown). To prevent large gaps from biasing the differences between individuals, the large repeat regions were removed between 306 bp and 502 bp when gaps were utilized as a 'fifth' state nucleotide.

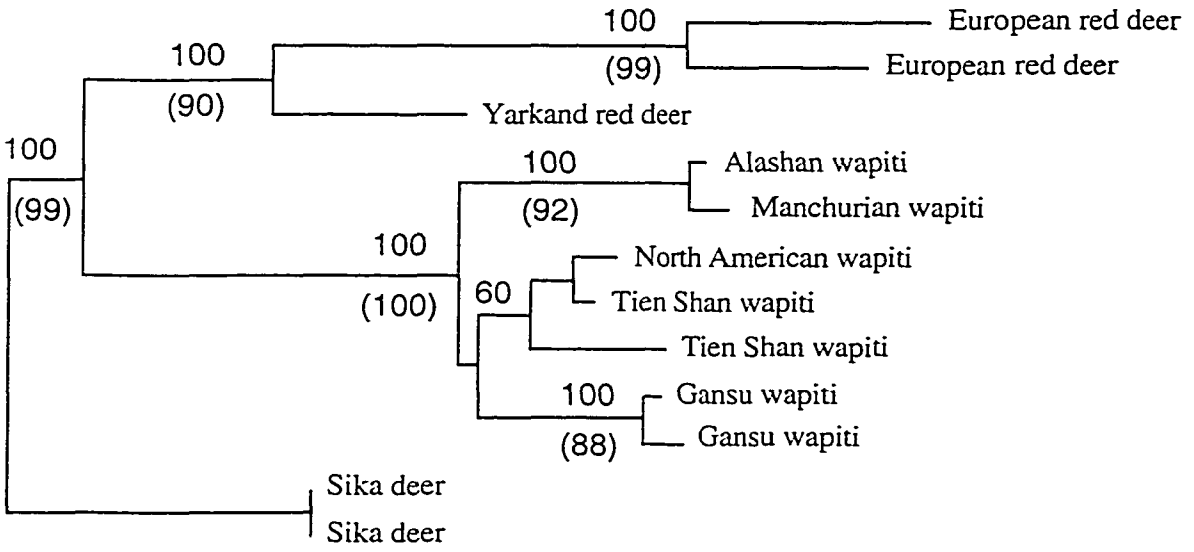

Figure 5-4. Phylogeny based on sequence differences from the cytochrome B region of mitochondrial DNA. The frequency of observing branching patterns using parsimony analysis are shown above each line and bootstrap values for 100 replicates are indicated in parenthesis. A polytomy was noted for the wapiti group in the bootstrap analysis.

The phylogeny constructed from the cytochrome B sequences included two Asian subspecies not analyzed for the control region. Thirty-one transitional and two transversional mutations occurred in the 367 bp sequences of which 26 characters were informative. An exhaustive search was used for all cytochrome B analysis. The distance tree based on Tajima and Nei with an uncorrected 'p' and assuming all sites equally mutate produced a phylogeny similar to the parsimony tree for the control region (Figure 5-4). A similar trichotomy of sika deer, wapiti and red deer was also shown in a cytochrome B tree rooted with fallow deer (*Dama dama*; Kuwayama and Ozawa, 2000). Distance values between the most related sequences are given in Table 5-2. The consensus of five equally parsimonious trees requiring 49 steps (CI = 0.816) had a topology identical to the distance tree (Figure 5-4). In the tree produced by maximum likelihood analysis (ML = 734.96) the Alashan/Manchurian group replaced the Tien Shan wapiti as a sister group to the Kansu wapiti (trees not shown) and increasing the weighting of transversional mutations to a maximum of 20:1 had no affect on the topology of the tree.

Table 5-3. Estimates of divergence within and among subspecies and species as determined from the mitochondrial sequences. The American subspecies include Rocky Mountain, Manitoban, and Roosevelt subspecies.

Control Region Comparison	(Years)	Suggested Taxonomic Division	Cytochrome B Comparison	(Years)
MacNeills/MacNeills	7,330	Population		
American[1]/American[2]	14,661			
Kansu/MacNeills	14,661		Manchurian/Alashan	13,600
Siberian/American	47,500		Tien Shan/American	40,850
Siberian/Manchurian	54,788			
American/Manchurian	58,516	Subspecies		
European/Barbary	74,492			
Kansu/Manchurian	73,135		European/European	95,350
European/European	128,771		Kansu/Alashan	95,350
European/Bactrian	184,745		European/Yarkand	177,100
Sika/American	208,559			
Bactrian/MacNeills	220,338			
Bactrian/Kansu	224,449			
Thorold's/Sika	226,864			
European/Sika	242,797			
European/American	243,262		Yarkand/Tien Shan	250,450
Thorold's/American	260,042			
Thorold's/European	266,779		Tien Shan/European	262,500

Nucleotide differences observed among the control region may identify three periods of divergence for cervid species (Table 5-3), where divergence estimates were

based on sequences that shared the fewest nucleotide differences in any pair-wise comparison. The oldest period (greater than 200,000 years) appears to be associated with the formation of species, and assuming a constant molecular clock, mtDNA lineages from red deer, wapiti, sika deer and Thorold's white-lipped deer diverged about 220,000 - 260,000 years ago. The most recent period (less than 20,000 years) appears to be associated with the development of variation within subspecies, and the variation within sequences from the MacNeills or Siberian subspecies was between 8,000 - 15,000 years. The time between 20,000 and 200,000 years may be associated with the formation of subspecies, where sequence divergence estimates from the control region suggested Asian subspecies separated between 40,000-75,000 years and as long ago as 129,000 years for European red deer. Sequence divergence estimates from the control region of the Bactrian red deer suggest that the subspecies diverged from the most related red deer (European) about 185,000 years ago, a time of separation that would have been enforced by glacial patterns.

Nucleotide differences observed between the cytochrome B sequences (Table 5-3) also suggest subspecies within Asia or Europe diverged between 13,600 - 95,350 years ago while Asian and European subspecies diverged from each other more than 262,500 years ago. The exception is the Yarkand red deer (*C. e. yarkandensis*) which has 177,100 years separating it from the European animals and 250,450 years separating it from the Asian animals. Like the Bactrian red deer, the Yarkand red deer was also separated from the European red deer during periods of glaciation.

Discussion

Interspecific Diversity

Using sequence divergence estimates to support taxonomic divisions appears to be difficult, even when comparisons are made to similar species that share an analogous distribution and history. The 5 % mtDNA sequence divergence between wapiti and red deer falls within the range of mitochondrial sequence divergences observed among subspecies and species, although comparisons are less than ideal when the mtDNA regions are not identical. Sequence divergence estimates from mtDNA of subspecies for the control region were 2.6% among caribou from Asia and North America (Dueck, 1998) and 3.9 % between moose from Sweden and North America (Mikko and Andersson, 1995), and 6-7 % for the complete mtDNA between black-tailed and mule deer of North America (Carr *et al.*, 1986). MtDNA sequence divergence estimates between species were only 2 % for the complete mtDNA of mule deer and white-tailed deer (Cronin, 1991) and

4 - 5 % for Asian and European sheep (Hiendleder *et al.*, 1998). Perhaps sequence divergence estimates as support for a species status are best understood when comparisons are made relative to subspecies and species from the same genus. For example the sequence divergence between red deer and wapiti was equivalent to the difference observed between sika deer (*Cervus nippon*) and Thorold's white-lipped deer (*C. albirostris*) or between either red deer or wapiti and sika deer.

Parsimony analysis of sequences from the control region suggests Thorold's white-lipped deer diverged before sika deer, followed almost immediately thereafter by wapiti and red deer. Patterns in antler development suggest the four point plan common to sika deer and Thorold's white-lipped deer appeared in the Villifranchian, the five point plan common to red deer at the beginning of the Günz, and the six point pattern typical of wapiti prior to the Illinoian or Riss glaciation (Geist, 1971). The loss of repeat units in the control region also reflects the sequence divergence pattern and may simply be co-incidental. Sequence divergence from the control region of mtDNA indicates the split among sika deer, red deer and wapiti lineages corresponds to the Riss or Salian glacial period that separated Europe from Asia. However, intraspecific comparisons of taxa over Europe indicated that divergence among lineages may not be connected to any particular cold period and that despite commonality among colonization routes each taxon is a unique case with its own history (Taberlet *et al.*, 1998).

That sika deer is a species distinct from *C. elaphus* is supported by genetic variation from this study, as well as from chromosomal variation (Fontana and Rubini, 1990), allozyme differences (Lowe and Gardiner, 1989), and the allelic distribution of microsatellites (Abernathy, 1994). Bartos and Zirovnicky (1982) claimed the chromosomal variation among sika deer was due to hybridization with related species, especially *C. elaphus*, although hybrid sika as indicated by the presence of wapiti mitochondrial haplotypes appear to be absent from indigenous populations of sika deer (Tamate and Tsuchiya, 1995; Kuwayama and Ozawa, 2000). Comparisons of red deer and sika deer vocalizations also provided striking differences (Long *et al.*, 1998) and to the casual observer in the field, there would appear to be three phenotypes of deer in areas where sika, red deer, and hybrids occur (Lowe and Gardiner, 1975).

Intraspecific Diversity of Red Deer

The distribution and physical similarities of *Cervus elaphus* were suggestive of a clinal distribution, where subspecies were partitioned into three broad geographic groups. However, the geographic divisions did not coincide with the division among mtDNA lineages, where animals from the central Asian group (wallichi) were assigned to red deer

(nominate) or wapiti (canadensis) groups. Subspecies included in the central Asian (wallichi) group were the Bactrian (*C. e. bactrianus*), Yarkand (*C. e. yarkandensis*), Kashmir (*C. e. hanglu*), Tibetan (*C. e. wallichi*), MacNeills (*C. e. macneilli*), and Kansu deer (*C. e. kansuensis*), where *yarkandensis*, *hanglu*, and *wallichi* were considered synonymous (Whitehead, 1972). Whitehead (1972) and Cockerill (1984) classified the Kansu and MacNeills deer as wapiti and assigned the other wallichi subspecies to the red deer group. Mitochondrial lineages also suggest the Bactrian and Yarkand deer belong to the red deer group while the MacNeills and Kansu deer belong to the canadensis group which is concordant with the classification proposed by Whitehead (1972) and Cockerill (1984). Further support for the classification of Bactrian and Yarkand subspecies as red deer was taken from the acoustic analysis of rutting vocalizations (Nikolski and Wallschlager, 1983), the presence of corronate antlers (Geist, 1971) and similarity of coloration (Flerov, 1952).

An improvement in scientific communication and taxonomy over the last century has seen a trend of uniting regional names under one synonym (Karl and Bowen, 1998). Reclassifying *Cervus* subspecies under one name highlights the remarkable similarity among the populations. For example, red deer from north-central Europe that included *C. e. scoticus* from the British Isles, *C. e. hippelaphus* from central Europe, *C. e. atlanticus* from Scandinavia, and *C. e. bolivari* from Spain were reclassified under the title *C. e. elaphus* (Groves and Grubb, 1987) on the basis of their similar markings and morphology (Flerov, 1952; Geist, 1971; Lowe and Gardiner, 1974). *C. e. maral* found in Hungary, Crimea, Asia Minor, Persia and the Caucasus, has a rutting call similar to *C. e. hippelaphus* (Nikolski and Wallschlager, 1983) and if synonymous with *C. e. hippelaphus* (Ellerman and Morrison-Scott, 1951) should also be reclassified as *C. e. elaphus*. A similar trend in categorizing populations into one subspecies has occurred in southern Europe, where Flerov (1952) lumped the deer from Sardinia, Corsica, North Africa, and southern Spain into the subspecies *C. e. corsicanus* because they all exhibit primitive antlers and coloration. Whether northern and southern European subspecies can be separated into two main lineages, as suggested from the classification noted above and the phylogenetic analysis from this study will be open to speculation until more sequences are analyzed.

Morphology, fossil records, and phylogenetic relationships have been used to determine the most ancestral form of red deer. The tendency for some red deer populations to retain spots in the adult summer pelage and for some males to have no bez or trez tine in their antlers may be perceived as an ancestral condition. Therefore, the Barbary deer (*C. e. barbarus*) would be regarded as deer having the most primitive

characters, although Groves and Grubb (1987) suggest that the primitive condition is more a response to poor foraging conditions. A close relationship between the North African Barbary deer and European red deer was shown in the phylogenetic analysis of mtDNA, which in combination with fossils collected from the area (Dolan, 1988), suggests red deer were able to cross the Levantine corridor from the Middle East and spread into North Africa during major retreats in sea levels. The suggestion that the Bactrian (*C. e. bactrianus*) and Yarkand deer (*C. e. yarkandensis*) represented red deer with the most ancestral traits (Groves and Grubb, 1987) was not supported by the mitochondrial phylogenies where they were identified as sister groups to the European red deer. Although Geist (1971) proposed the Bactrian and Yarkand red deer had probably dispersed eastward from Europe during mid-Pleistocene times and survived in central Asia in areas largely untouched by the ice sheets of the Riss and Würm glaciation, the estimates of sequence divergence suggest the dispersal was more likely to be westward from Asia.

Intraspecific Diversity of Wapiti

Although brief descriptions are available for the wapiti group that occupy China and Russia (Lydekker, 1893; Dolan, 1988), their subspecific distinctions have been debated. Mitochondrial lineages have a north - south division that concurs with the classification presented by Tate (1947) and Harper (1945) who grouped wapiti found in northern Korea, Mongolia, Manchuria, and Siberia together as Manchurian wapiti (*C. e. xanthopygus*) and grouped wapiti found in south-central China together as Kansu wapiti (*C. e. kansuensis*). The similarity among the northern group is strengthened by independent observations of morphology that suggested the Tien Shan (*C. e. songaricus*) wapiti were synonymous with Siberian (*C. e. sibiricus*) wapiti (Heptner *et al.*, 1961; Bobrinsky and Flerov, 1934) and Siberian were synonymous with North American wapiti (*C. e. nelsoni*; Geist, 1971). A similarity in antler configuration was also noted by Lydekker (1893) who thought it would be impossible to tell the difference between antlers shed by Tien Shan or North American wapiti. Acoustic analysis of rutting calls also identified a similarity between Manchurian and Siberian wapiti, although Nikolískii *et al.* (1987) did not believe it was sufficient to admit the populations to the same subspecies.

The trend to reclassify populations under one subspecific label can also be observed in North America. Lumping the Eastern (*C. e. canadensis*), Merrium (*C. e. merriami*), Rocky Mountain (*C. e. nelsoni*) and Manitoban (*C. e. manitobensis*) populations found in North America under *canadensis* (Groves and Grubb, 1987) is supported by this work as well as recent studies by Schonewald (1994), Polziehn *et al.* (1998), and Polziehn *et al.* (in press). MtDNA sequence differences were unable to

differentiate wapiti sampled from the historic range of the Eastern wapiti in Ontario from Rocky Mountain or Manitoban wapiti, whereas Roosevelt wapiti (*C. e. roosevelti*) from Vancouver Island and Tule wapiti (*C. e. nannodes*) from California appear to be distinct subspecies (Denome, 1998; Polziehn *et al.*, 1998). Determining the genetic variation of the Eastern and Merrium wapiti will be difficult as both subspecies are extinct and any analysis will rely on a few archived specimens.

Hybridization

The proximity of Asian subspecies to each other creates a possibility of hybridization and complicates the task of classifying populations. For example, the Alashan wapiti (*C. e. alashanicus*) range is found between the ranges of the Kansu and Manchurian subspecies, and the Alashan wapiti has been considered synonomous with the Kansu subspecies (Groves and Grubb, 1987). Specimens from the Shansi province of China also suggest an intergradation between Kansu and Manchurian wapiti (Groves and Grubb, 1987). However, sequence differences from the mtDNA suggests the Alashan wapiti are distinct from both Kansu and Manchurian wapiti and that integration may not be common between north and south China. A similarity in coloration between the Kansu and McNeills wapiti (Harper, 1945) also may suggest hybridization is occurring between these subspecies or that they are synonymous (Whitehead, 1972), which concurs with the sequence divergence estimate of 15,000 years that implies Kansu and McNeills wapiti have recently diverged or that they are also synonymous.

Hybridization between subspecies is also a problem for defining subspecies within North America. Once thought to be the home of Roosevelt wapiti (*C. e. roosevelti*), the Olympic Peninsula of Washington currently is believed to have a mixture of Roosevelt and Rocky Mountain animals. Sequence data from the control region confirms earlier observations by Polziehn *et al.* (1998) which discovered a proportion of non-Roosevelt mitochondrial haplotypes within the population. The presence of these animals may be an artifact of relocated Rocky Mountain wapiti from Yellowstone National Park or a natural occurrence as both Rocky Mountain and Roosevelt wapiti were indigenous to Washington (Bryant and Maser, 1982).

Conclusions

Using the measure of sequence divergence to distinguish taxonomic divisions is hardly a novel idea, and is likely to be re-evaluated as sequence information accumulates. Translating the genetic differences into years of separation is an extension of this idea that allows comparisons to be drawn between glacial and molecular patterns. From

comparisons of the control region of mtDNA, sequence divergence among cervid species appears to have taken place more than 220,000 years ago while subspecies commonly placed between 50,000 and 100,000 years ago. Divergence estimates from mtDNA lineages of Asian and North America subspecies were often less than 20,000 years (Polziehn *et al.*, 1998) suggesting that these subspecies are recently evolved or perhaps unsuited to a subspecies status.

Comparisons of mitochondrial DNA variation have been the method of choice to determine phylogeography, an area of study concerned with the processes governing the geographical distributions of lineages, particularly those at an intraspecific level (Avise, 1998). However, disagreement of the taxonomic classification for the sea turtle (Karl and Bowen, 1999), and brown bear (Waites *et al.*, 1999) highlights the need to accrue information from mtDNA, nuclear DNA, and morphological sources. Concordance among the evolution of independent characters makes the partitioning of taxa straight forward or at least less controversial (Grady and Quattro, 1999). Fortunately, the divergence of mitochondrial lineages noted for sika deer, red deer, and wapiti is in agreement with geographical, morphological, and behavioral distinctions. In addition, variation among sequences as determined from this study demonstrate wapiti were equivalent to red deer and sika deer in their divergence and are worthy of the species status. Nevertheless, until additional molecular information comes available for *C. elaphus* subspecies from central Asia, evidence from mtDNA should be viewed as only one step toward the re-instatement of *C. canadensis* for wapiti subspecies.

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Chapter 6

Summary

Mitochondrial and Microsatellite DNA

More than 600 studies have used the differences found among mitochondrial DNA sequences to determine phylogenetic relationships among organisms since mtDNA was first assayed using restriction enzymes (Brown & Vinograd 1974) and many have focussed on the control region of mtDNA as it contains regions that are highly variable. Genbank, an international repository for genetic sequences, currently contains more than 12,000 sequences from this region alone. Although rates of mutation from the control region appear to vary among classes of vertebrates, the relative lack of variation observed among North American wapiti populations (Chapter 2) suggests that extrinsic factors have likely contributed to the low levels of genetic diversity, especially when other large ungulates express high levels of variation (Ellsworth *et al.* 1994, Matthee & Robinson 1999).

Microsatellite markers also have gained popularity because they have revealed high levels of variation (large number of alleles) and because the same suite of markers can often be surveyed across related species. Furthermore, finding a variable set of markers in most species usually requires only a small number of loci to be screened. However, more than 200 published microsatellite loci have been assayed in red deer or wapiti and only a handful have been shown to be variable in wapiti populations (Talbot *et al.* 1996; Wilson *et al.* 1997; Slate *et al.* 1998; Roed & Midthjell 1999). At present, the typical number of microsatellite markers used in studies will vary from 8 to 18 depending to a large extent on variability of the markers and the nature of the study. Even though twelve of the most variable microsatellite markers were employed to determine the genetic variation among wapiti populations (Chapter 3), they revealed a relative lack of variation among wapiti. When microsatellite markers were employed to measure genetic diversity among other large ungulates in North America, bison and moose also expressed low levels of genetic diversity while caribou and white-tailed deer showed high levels of variation (Wilson *et al.* 1997, Broders *et al.* 1999; Zitlau *et al.* in press).

In response to criticisms that studies were overly reliant on a single gene system, in particular mtDNA, several recent phylogeographic, genetic diversity, and population studies have incorporated both mitochondrial and nuclear DNA information. Congruence between nuclear and mitochondrial evidence as support for the distinction among populations, subspecies and species usually produces little argument over the divisions in classification or in the recognition of ESU (evolutionary significant units). Fortunately, North American wapiti populations identified as unique using the control region of mtDNA (Chapter 2) were also identified as unique using genotype distributions based on microsatellites from the nuclear genome (Chapter 3). Crandell *et al.* (2000) emphasized the importance of recognizing phenotypic variation in conservation programs that would preserve important adaptive characters and their underlying genetic variation. Unfortunately, when neutral loci such as the mitochondrial control region and microsatellites are surveyed, they do not indicate the variation that may develop in genes under the influence of natural selection.

Classification

Population level

Although North American wapiti populations appear to be stable or increasing, they have little genetic diversity at mitochondrial or microsatellite markers (Chapters 2 and 3). The reduced level of diversity does not appear to manifest itself in the form of reduced fertility or physical defects, and though genetic diversity may be low, the ability to assign most individuals to their respective population using the distribution of microsatellite markers suggests some level of population structure. The low genetic variation unfortunately makes the assignment test of limited use for forensic applications. Geographic barriers, predator distribution, forage availability, and hunting are all factors that appeared to contribute to population distinction (Chapter 3), whereas dispersal, either natural or through relocations, is a likely explanation for the uniformity of microsatellite genotype distributions observed among mountain park populations.

The inability to detect herd structure in Jasper National Park (Chapter 3) likely reflects the increased dispersal of individuals in large mature populations (Albon *et al.* 1992; Bender & Haufler 1999) and raises the question of how to delimit wapiti populations. When populations are isolated, such as the wapiti located at French River, Burwash, and Vancouver Island, their range is easy to define. When populations are not so distinct, it is important that wildlife biologists manage for a larger area otherwise they will be trying to regulate a population that consists of herds that move in and out of

their control. For example wapiti in the Rocky Mountains appear to migrate among Banff, Kootenay and Yoho but not Jasper National Park (Chapter 3), which suggests that the Jasper population could be excluded from a management plan that included the other three parks.

Subspecies level

Ball and Avise (1992) proposed that 'subspecies names should be reserved for the major subdivisions of gene pool diversity within species where evidence for phylogenetic distinction among putative subspecies must come from the concordant distributions of multiple independent, genetically based traits.' Keeping this in mind, the distribution of microsatellite genotypes and mitochondrial haplotypes noted in Chapters 2 and 3 support the contention that Rocky Mountain and Manitoban wapiti should be considered a single subspecies (Bryant & Maser 1982). In contrast, the unique mitochondrial lineages and microsatellite distributions observed for the isolated populations on Vancouver Island and California suggest that the Roosevelt and Tule wapiti, respectively, represent distinct subspecies. A reclassification of wapiti to include at most three subspecies in North America would be in line with the suggestion to reclassify the four European red deer subspecies as one (Groves & Grubb 1987). A reduction of the number of subspecies was certainly a trend of the 1990's, although Karl and Bowen (1998) suggested this soon may end if conservation plans emphasize taxonomic status.

An overall lack of variation in mitochondrial and microsatellite genotypes across the range of wapiti suggests changes in climate and available habitat during the Pleistocene were largely responsible for the reduced variation among wapiti and, therefore, the low genetic variation is not strictly due to recent human-induced bottlenecks and relocations. Ramey (1995) proposed population fluctuations caused by changes in climate and vegetation, coupled with stochastic extinction and subsequent recolonizations, may have severely limited mtDNA variation among desert-dwelling mountain sheep. Species that suffer large fluctuations in population size, even those with high gene flow, will have low sequence divergence among haplotypes (Avise *et al.* 1988). Therefore recurrent population explosions and crashes may provide one possible explanation for the low diversity in North American wapiti. Consequently, relocating wapiti to areas that are currently populated will likely have little effect on changing the overall genetic diversity, however, precaution should be taken to conserve the unique Roosevelt and Tule populations as well as to consider any local adaptations.

Species level

Most of the species and subspecies or races of red deer or wapiti have been named on the basis of antler, coat, and body characteristics. Furthermore, relatively few specimens have been examined, the criteria used to separate taxa has never been quantified, and the range of variation has not been measured (Lowe & Gardiner 1989). Lydekker noted an overlap in characters among races of Europe and Asia as early as 1898, and this raised the question of whether or not the character distributions were typical of a cline. Although several recent studies were unable to distinguish red deer, wapiti, and the closely related sika deer (Emerson & Tate 1993, Cronin *et al.* 1996), the discovery of a significant level of genetic variation among their mitochondrial sequences, as noted in Chapter 4, suggested a long period of separation among the three groups. To date, the mitochondrial DNA survey of wapiti and red deer in Chapter 5 represents the most comprehensive genetic survey of *Cervus elaphus.* The geographic distribution of mitochondrial haplotypes appears to be defined by the Himalayan Mountains and Gobi Desert. The separation between mitochondrial haplotypes concurs with the red deer and wapiti division defined by Cockerill (1984) and Whitehead (1972) and discredits the classification suggested by Groves and Grubb (1987).

The suggestion that sika deer may be more related to wapiti was based on mitochondrial sequence comparsions and the ability to interbreed (Chapter 4). Similarities among sequences from the control region and cytochrome b (Kuwayama & Ozawa 2000) of sika, red deer, and wapiti stress the importance of including closely related species in phylogenetic comparisons, as the addition of Thorold's white lipped deer in the outgroup produced a phylogeny that agreed with current taxonomy (Chapter 5). The ability of sika deer and wapiti to hybridize also suggests a close relationship between the two species, however Nagata *et al.* (1998) and Kuwayama and Ozawa (2000) discovered no hybrids in their studies. Absolute reproductive isolation cannot be used as the criterion of specific distinctness as occasional hybridization and even the production of fertile hybrid offspring occurs between species that are normally genetically isolated from one another and that maintain distinct and different gene pools (Mayr 1942; Dobzhansky 1951, White 1978).

If one role of classification is to reflect the evolutionary divergence among species, the current classification of *Cervus elaphus* fails to meet this expectation. The divergence among the mitochondrial lineages of red deer and wapiti subspecies is equivalent to the divergence between the related Thorold's white lipped deer and the sika deer (in the range of 200,000 –300,000 years), while other subspecies within the

red deer or wapiti group are distinguished by divergence estimates in the 50,000 - 100,000 year range or as little as tens of thousands of years. The former division is an example of under splitting and the latter of over splitting *C. elaphus*. Although the conclusion from this thesis is based on a single gene, I would recommend the reinstatement of the classification of *C. canadensis* for wapiti. As most deer in central Asia are under the threat of extinction, from a conservation standpoint I foresee a positive outcome by acknowledging the divergence between red deer and wapiti groups.

Future

One of the most obvious directions that should be pursued is to construct a phylogeny for red deer and wapiti based on nuclear genes. Congruence between the nuclear and mitochondrial trees would only serve to validate the conclusions presented here. Additional acoustic analysis of the male mating calls from central Asian subspecies would also be useful, as this character has been shown to have a genetic component (Long *et al.* 1998) and it can serve as an isolating mechanism between red deer and wapiti. Although the SRY gene was not found to be informative for discerning wapiti and red deer (Addendum), additional regions of the Y chromosome should be considered, as they would provide a means for following paternal lineages.

As was performed among North American wapiti subspecies, a similar comparison of mitochondrial haplotypes and microsatellite based genotypes should be completed for Asian and European subspecies. Asian wapiti subspecies would benefit from a molecular study as hybridization events and short periods of divergence confuse the classification of populations. One difficulty with this study or the one mentioned above is that samples are nearly impossible to obtain from Asia, as most wapiti populations face extinction and are listed on the CITES appendix. A survey of northern and southern European populations also would help to substantiate the suggestion to reduce the number of European subspecies categories. From the survey of subspecies noted in Chapter 5, the separation of red deer lineages hints at a northern and southern separation.

On a more molecular note, questions can be raised as to why some microsatellite loci appear to remain variable and whether the sequences or location of the markers can be used to predict the usefulness of the marker. As the mapping projects for sheep, cattle, deer, and pig become complete, comparisons among marker sequences and locations should be enlightening. All current bovine and ovine marker locations have been mapped to red deer chromosomes.

On a phylogeographic note, questions can be asked as to whether general patterns for the distribution of species can be observed. For nearly as many studies that show similar patterns of molecular evolution and distribution among species, as many can be found that show no relationship. However, few comprehensive studies are available, and perhaps patterns will become more evident when studies compare organisms that evolve together. Several studies show parasite-host or symbiotic co-evolution, but few studies look at the co-evolution of competitor species or the co-evolution of species in an ecosystem ... an ecophylogeographic approach. In other words, how does the distribution of species affect the distribution of other species, and how is this reflected in their phylogeography. As the past ten years has shown, an increase in the collaboration between ecologists, taxonomists, and population geneticists, in the future should reveal even more insights from interdisciplinary fields.

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Chapter 7

Addendum - Comparative sequences from the SRY gene from North American ungulates

Introduction

The *SRY* gene, located on the Y chromosome, is required for male sexual differentiation in mammals (Sinclair *et al.* 1990). The *SRY* locus shares the attributes of non-recombination and clonal inheritance with mitochondrial DNA (mtDNA), although both an absence and multiple copies of the *SRY* gene have been observed in rodents (Pamilo and O'Neill, 1997). The protein encoded by the *SRY* gene contains a high mobility group (HMG) domain of approximately 78 amino acids that is characteristic of DNA binding proteins (Sinclair *et al.* 1990). Among distantly related species, similarity in the HMG domain can be greater than 97 % (Whitfield *et al.* 1993). While the conserved nature of the HMG domain underlies it function, no role has been ascribed to the rapidly evolving C- and N- terminal regions where heterogeneous lengths are common to the N-terminal region (Whitfield *et al.* 1993). Although the *SRY* gene provides the opportunity to study the evolution of both recent and distantly related species, phylogenetic inferences have only be drawn for mice (Lundrigen and Tucker, 1994), humans (Whitfield *et al.* 1995), and a small number of widely diverged mammals (Margarit *et al.* 1998).

In this study, comparisons of the *SRY* locus were used to support phylogenetic divisions within the order Artiodactyla. The three families of the Artiodactyla found in North America include the Antilocapridae, Bovidae, and Cervidae, all which owe their origin to ancestors that crossed the Bering land bridge from Asia. The Antilocapridae are represented by only one extant species, the pronghorn (*Antilocapra americana*) that are endemic to North America. Although the modern pronghorn has existed for 100,000 years, fossils date back to the Miocene for this family (Kurten and Anderson, 1980). The widely distributed Bovidae are divided into five subfamilies, two of which are the Bovinae and Caprinae. The Bovinae include the North American bison (*Bison bison*) and the European wisent (*B. bonasus*), which are recognized as different species. The Caprinae in North America are represented by bighorn sheep (*Ovis canadensis*) and mountain goat (*Oreamus americanus*), which have a limited distribution along the Rocky Mountains and fossil records in North America that predate the last ice age (Kurten and Anderson, 1980).

The Cervidae are divided into four subfamilies of which two are the Cervinae and Odocoileinae. The wapiti (*Cervus elaphus*) is the only member of the Cervinae in North America, and currently consideration is being given to reclassify the conspecific North American wapiti and the European red deer (Polziehn *et al.* 1998, Polziehn and Strobeck, submitted). White-tailed deer (*Odocoileus virginianus*), mule deer (*O. hemionus hemionus*) and black-tailed deer (*O. h. columbianus*) belong to the subfamily Odocoileinae and are endemic to North America, while moose (*Alces alces*) and caribou (*Rangifer tarandus*) have a holarctic distribution and their arrival prior to the last glaciation has not been noted in the North American fossil record (Kurten and Anderson, 1980).

Phylogenetic analysis of the subfamilies within the class of Artiodactyla has utilized genetic information from allozymes (Baccus *et al.* 1983; Emerson and Tate, 1993), mtDNA (Miyamoto *et al.* 1990; Cronin, 1991; Allard *et al.* 1992; Gatesy *et al.* 1992; Polziehn and Strobeck, 1998), repetitive DNA (Lima-de-Faria 1984), and nuclear genes (Cronin *et al.* 1996). These genetic studies were able to conclude that most subfamilies and families are monophyletic, but often could not resolve relationships among higher taxonomic ranks. At a species and subspecies level, mtDNA data has been used to construct phylogenies for many North America ungulates, however, greater confidence in the mitochondrial based trees would be achieved if they showed congruence with nuclear based phylogenies. Therefore, sequences from the *SRY* locus from ungulates of North America will be used to support existing molecular based phylogenies.

Materials and Methods

Total DNA was isolated from tissue samples using the Qiagen QIAmp tissue isolation kit (Chatsworth, CA). Tissue samples obtained from the national parks were collected from animals killed by motor vehicles, while those obtained from Fish and Wildlife offices were made available from hunter kills or accidental deaths. Park wardens, respectively from Banff and Riding Mountain National Parks collected tissue from Rocky Mountain (*Cervus elaphus nelsoni*) and Manitoban wapiti (*C. e. manitobensis*). Tule wapiti (*C. e. nannodes*), European red deer (*C. e. elaphus*), and sika deer (*C. nippon*) tissue samples were supplied by the Forensics Laboratory of the United States Fish and Wildlife in Oregon. San Diego Zoo supplied hair samples for the white lipped deer (*C. albirostris*). Park wardens from Banff, Elk Island, Glacier, and Wood Buffalo National Parks, respectively, collected muscle tissue from mule deer (*Odocoileus hemionus hemionus*), moose (*Alces alces*), and mountain goat (*Oreamus americanus*), and blood from bison (*Bison bison*). Samples of bighorn sheep (*Ovis canadensis*), pronghorn

(*Antilocapra americana*), and white-tailed deer (*virginianus*) were supplied by Alberta Fish and Wildlife, and samples of caribou (*Rangifer tarandus*) and black-tailed deer (*O. h. columbianus*) were provided by British Columbia Fish and Wildlife.

Sequences for the *SRY* locus for *Ovis aries* (Z30265), *Bos taurus* (Z30327), *Bison bonasus* (Z30321) and *Capra hircus* (Z30646) were taken from Payen and Cotinot (1994); *Canis familiaris* (U15160) from Meyers-Wallen *et al* (1995) and *Lama quanicoe* (U66068) from Meyers-Wallen *et al.* (unpublished); *Gazella dorcas* (AJ003127) from Margarit *et al.* (1998); *Equus caballus* (Z26908) from Griffiths and Tiwari (1993); and *Cervus nippon* (AB004667) from Takahashi *et al.* (1998). Accession numbers are noted in brackets.

Amplification of the *SRY* locus employed primers designed to be internal to the Bovidae sequence described by Payen and Cotinet (1994) and are as follows for the HMG domain: CST 163 5'GTGAAGCGACCCATGAACGCATT3' and CST 164 5'AGC ATTTTAGCCTTCCGACGAGG3' and are as follows for the N-terminal: CST 1549 5'TGCTATGTTCAGACTATT3' and CST 1550 5'CGTTCATGGGTCGCTTCAC3'. The PCR protocol included denaturing at 94 ^{o}C for 1 min., thirty-five cycles of 94 ^{o}C for 30 s., 56 ^{o}C for 20 s, and 72 ^{o}C for 7 s., followed by a final extension at 72 ^{o}C for 30 s. Primers were synthesized in the lab on a 391 Applied Biosystems PCR-mate, and amplifications were performed on a 9600 Perkin Elmer thermocycler. Purification of the amplified products required separation on a 2 % agarose 0.5 X TBE gel using electrophoresis, excision of the desired bands, and the application of Qiagen QIA-quick gel extraction kit to elute the DNA. Sequencing reactions were performed as described in the dRhodamine cycle sequencing kit by Applied Biosystems, Inc. Sequences were collected on a 377 ABI Automated sequencer and analyzed using ABI 377 Sequencer software.

Consensus sequences and alignments were constructed using the computer program Sequence Editor. Phylogenies were constructed from the sequences using parsimony and maximum-likelihood options of the computer program PAUP 4.0b (Swofford 1993). Trees were rooted using sequences from oders Artiodactyla (*Llama guanicoe*), Carnivora (*Canus familiaris*) and Persidactyla (*Equus caballus*).

Results

Including primers, the amplified products for the HMG domain of the *SRY* locus were 236 bp. The sequences for the fifteen ungulates, representing eleven species and

seven subspecies are summarized in Table 7-1. Thirty nucleotide sites varied among the Artiodactyla sequences, whereby substitutions resulted in non-synonymous changes at fifteen sites, three character states at five sites, and charge changes in amino acids at nine sites. Seven, six and 17 nucleotide substitutions occurred in the first, second, and third position of a codon, respectively.

No unique sites characterize the families Bovidae, Cervidae, or Antilocapridae, but unique sites that distinguished their subfamilies were listed in Table 7-2. Within the family Bovidae, five synonymous and five non-synonymous substitutions were observed, and within the family Cervidae, two synonymous and four non-synonymous substitutions were observed. Generally, closely related genera cannot be identified by diagnostic substitutions, however, three substitutions in *Alces* appear to separate this genus from other genera of Odocoileinae and substitutions at site 50 were able to separate *Cervus elaphus*, *C. nippon*, and *C. albirostris*. No nucleotide substitutions were observed among four North American *C. elaphus* subspecies that were denoted as *C. e. canadensis* (data not shown).

Exhaustive searches using PAUP were employed to construct both parsimonious and maximum likelihood trees from sequence data from the HMG region. Parsimony analysis produced three equal length trees, regardless of whether characters were equally weighted or non-synonymous mutations were weighted more than synonymous mutations. As transversional to transitional mutations were weighted beyond a ratio 5:1, the root of the tree moved to the node between *Cervinae* and *Odocoileinae*. A consensus tree and bootstrap values for 100 replicates are shown in Figure 7-1. Maximum likelihood analysis produced a tree that required 56 steps, had a log likelihood of -558.830, and had the same topology as shown in the parsimony analysis.

Sequences were secured from seven species for the N' terminal region, however only in the 3' to 5' direction (Table 7-3). Including primers, amplified products for the N' terminal region of antelope, bison, red deer, wapiti and white-tailed deer were 182 bp in length and 249 bp in length for moose. When the sequences were compared to 5 additional known sequences, 38 sites were found to vary, four of which had more than two character states and one that was a long insertion. Sequence insertions and deletions were noted in the SRY gene (Margarit *et al.* 1998) although most were not as long as that observed within the 5' N terminal region of the SRY gene of moose. Unique sites that can discriminate between the families and subfamilies of Artiodactyla are given in Table 7-2. Within the subfamily Bovinae, four sites were unique to *Bison* and *Bos*, one site was shared among *B. bonasus* and *B. bison*, and one site was unique to *B. bison*. Four mutations identify the family Cervidae, and one site was unique to both the Odocoileinae

and Cervinae. There were four mutations unique to *Alces*, but no sites unique to *Odocoileus* or *Rangifer*, and likewise, there were no sites unique to *Cervus canadensis* or *C. elaphus*. Parsimony analysis produced 11 equal length trees (157 steps and CI = 0.911) of which one was equivalent to the tree produced by maximum likelihood. The maximum likelihood tree had a similar topology to the phylogeny based on the HMG region (tree not shown).

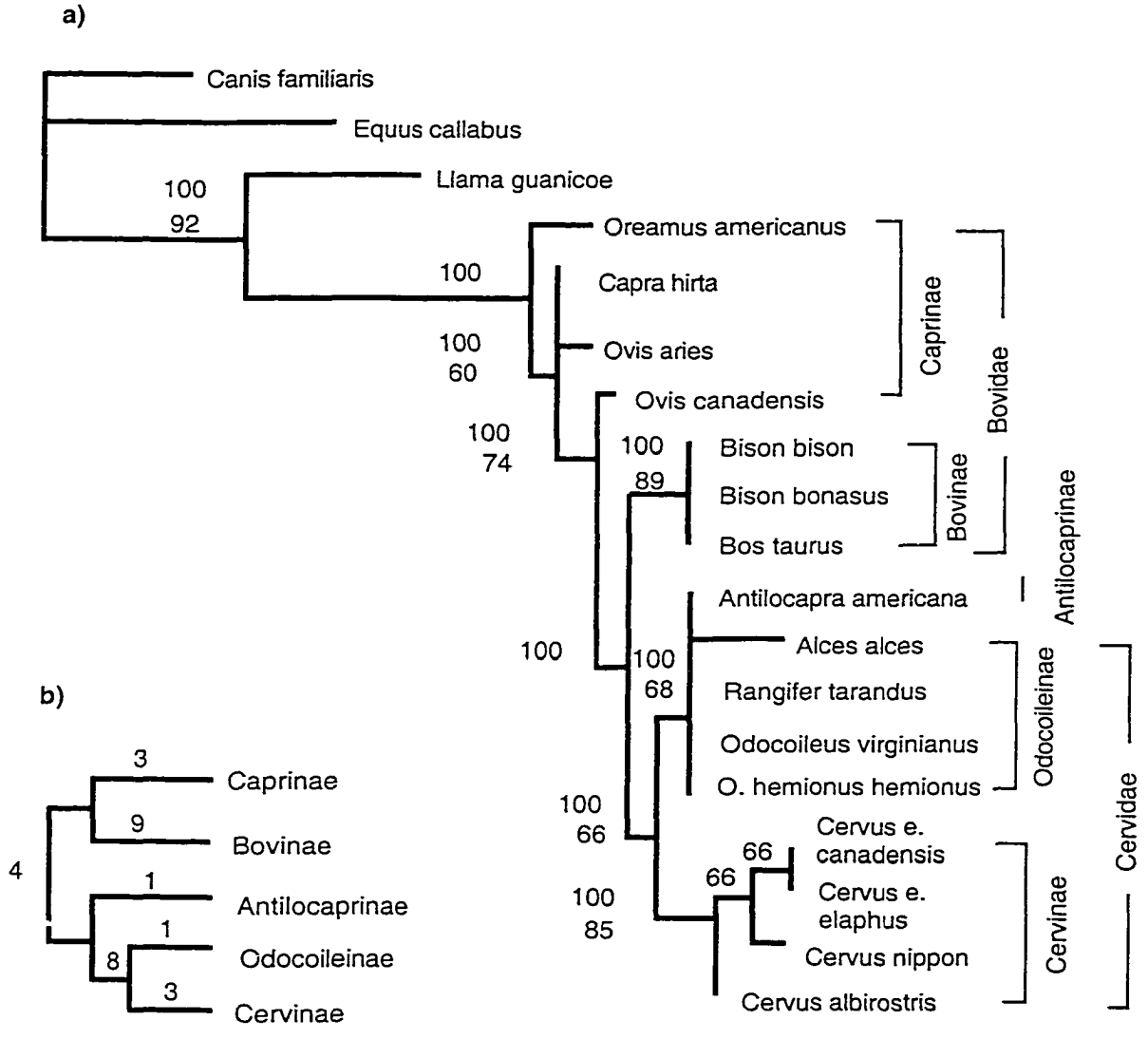

Figure 7-1. Phylogenetic relationships among Artiodactyla subfamilies as based on the SRY gene. a) Consensus of the three most parsimonious trees where bootstrap values are noted at each node. b) Parsimony tree constructed from characters given in Table 7-2, where values represent the unique distinguishing character states between nodes (33 steps and CI = 0.88).

Discussion

Sequence diversity from the *SRY* gene has been used to determine population, species, and generic relationships. For example, Lundrigan and Tucker (1994) found up to 8.16% sequence divergence between species of mice (*Mus*), and were able to divide the paternal ancestry into Palearctic and Oriental groups. However, the variation does not appear to be adequate to resolve evolutionary relationships much below the subfamily level in Artiodactyla, regardless if the HMG or the 5' region was employed.

Mutations that characterize subfamilies and genera were observed, although additional samples will need to be surveyed to determine if the nucleotide substitutions are truly diagnostic. Observations from the *SRY* gene in this study, satellite DNA (Modi *et al.* 1996), mtDNA (Irwin *et al.* 1991; Gatesy *et al.* 1992) and nuclear DNA (Cronin *et al.* 1996) support a monophyletic clade for *Bos* and *Bison*. Furthermore, a nucleotide substitution was observed at the beginning of the HMG sequence that distinguished *Bison* from *Bos*, which may be useful for following paternal contributions among bison herds derived from founding stock that have had historic contact with cattle (Polziehn *et al.* 1995). The monophyletic subfamilies of Cervinae and Odocoileinae observed in the *SRY* were also consistent with phylogenies from mtDNA (Polziehn and Strobeck, 1998; Douzery and Randi, 1997; Irwin *et al.* 1991), nuclear DNA (Cronin *et al.* 1996), and morphology (Groves and Grubb, 1987). With regards to relationships among *Cervus* species, variation within the *SRY* gene supports the current taxonomic division that recognizes *C. nippon* as a species separate from *C. elaphus* (Groves and Grubb, 1987; Polziehn and Strobeck, submitted). However, no diagnostic markers were observed from the SRY gene to separate European red deer (*C. elaphus elaphus*) and American wapiti (*C. e. canadensis*).

Although molecular comparisons were unable to resolve the relationship among the families Cervidae, Antilocapridae, and Bovidae using satellite DNA (Modi *et al.* 1996) and mtDNA (Kraus and Miyamoto, 1991), the variation within the SRY gene, satellite DNA (Lee *et al.* 1997), DNA/DNA hybridization (Douzery *et al.* 1995), and the K-casein gene (Cronin *et al.* 1996) supports a division between Cervidae and Bovidae. The relationship between these two families and Antilocapridae continues to perplex taxonomists, although a preference to group Antilocapridae with Cervidae was shown in this study, as well as in the comparisons of mtDNA sequences (Douzery and Randi, 1995; Irwin *et al.* 1991) and morphological characters (Groves and Grubb, 1987; Leinders and Heintz, 1980).

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Table 7-1. Comparison of HMG nucleotide sequences and predicted amino acid sequences for sixteen species of mammals, where the most common amino acid sequence is listed below the nucleotide sequences. The underlined sequences indicate regions of the primers. The effect of nucleotide substitutions on their encoded amino acids are as follows: substitutions enclosed in a box indicates a charge change, bolded indicates replacement with equal charge, italicized indicates hydrophilic change, and underline indicates hydrophobic replaced with hydrophobic amino acid.

```
                                                                                                  60
CATTGTGTGG TCTCGTGAAC GAAGACGAAA GGCGGCTCTT GAGAATCCCA AATTGCAAAA
.......... .......... .......... ..T......A .......... ..........
.......... .......... .......... ..T......A .......... ..C.......
.......... .......... .......... ..T......A .......... ..........
.......... .......... .......... ..T......A .......... ..A.......
.......... .......... .......... ..T......A .......... ..A..[A]....
.......... .......... .......... ..T......A .......... ..A..[A]....
.......... .......... .......... ..T......A .......... ..A.......
.......... .......... .......... ..T......A .......... ..A.......
.......... .......... .......... ..T......A .......... ..A.......
.......... .......... .......... ..T......A .......... ..A.......
.......... .......... .......... ..T......A .........[G] ..A.......
.......... .......... .......... ..T......A .........[C] ..A.......
.......... .......... .......... ..T......A .......... ..A.......
......A... G.......T. A...G..... ..T......A .......... ..A....G..
...G...... ........T. AC..G..C.. ..TC.....A .........C ..C.......
.T.G...... .....C..TC A...G..C.. .AT......A .........C ..A.......
   I  V  W    S  R  E    R  R  R    L  V  A  L    V  N  P    K  M  Q
```

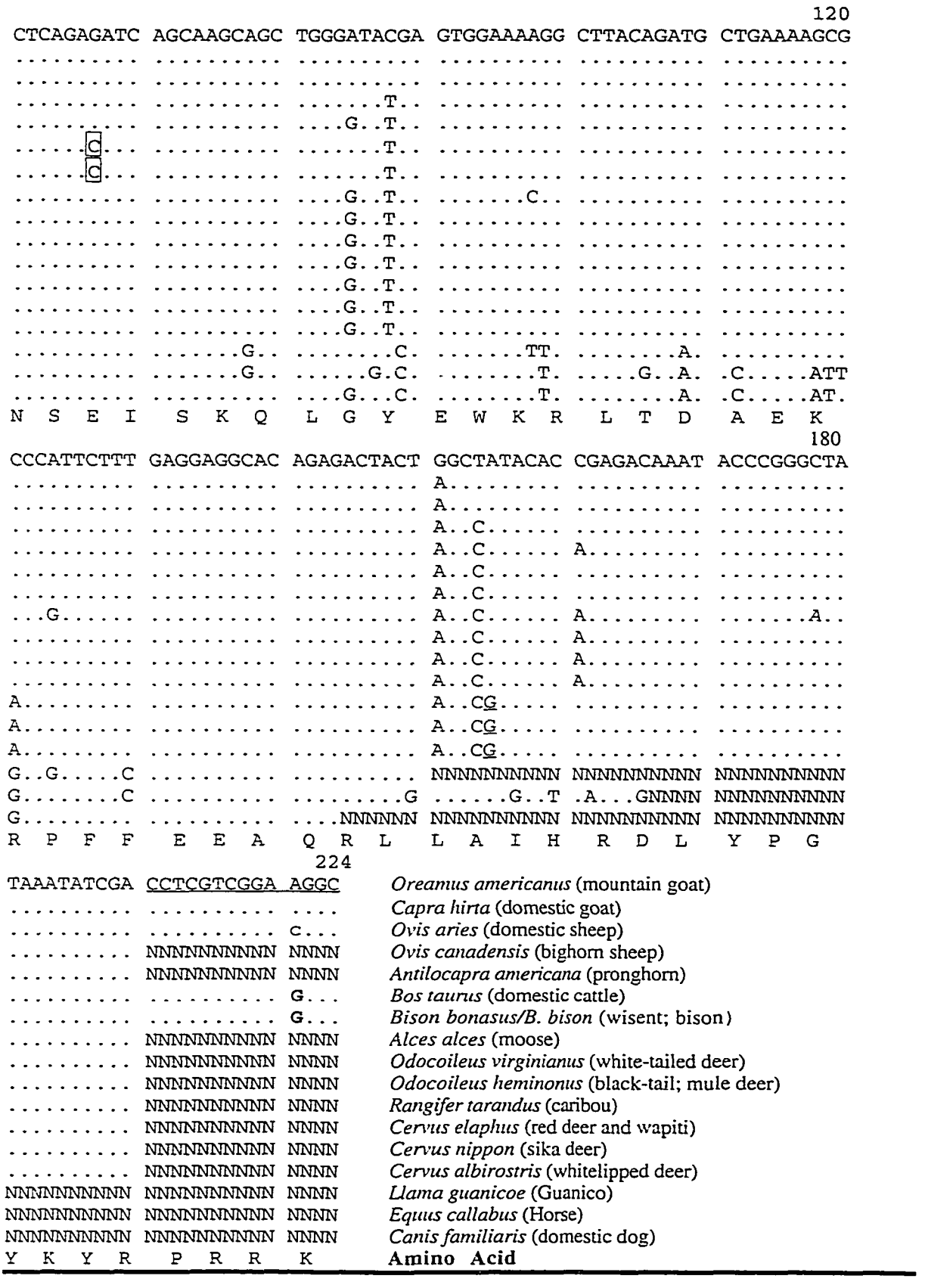

Table 7-2. Summary of variable sites unique to families and subfamilies of Artiodactyla for regions of the SRY gene. Sequence information that is not available is indicated with a (?). Families are identified as A = Antiocapridae, B = Bovidae, C= Cervidae and D = Camelidae.

Classification			HMG Region									5'N Region																			
	Subfamily	Genus	53	60	67	85	88	121	155	161	221	141	120	118	111	104	101	98	89	84	83	70	61	58	57	35	26	15	11
A	Antilo-caprinae	*Antilocapra*								A			G									C		G	C	C			G
B	Caprinae	*Capra*	T									A	G					T		A									
		Ovis	C/T									A	G					T		A									
		Oreamus	T									?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
		Gazzela	?	?	?	?	?	?	?	?	?		G							A			G						
	Bovinae	*Bos*		A	A						G					C			A		A		G				C		
		Bison		A	A						G[1]					C			A		A		G				C		
C	Odocoil-einae	*Odocoileus*				G	T			A			C	A	A		C					T		G	C	C		C	G
		Alces				G	T			A			C	A	A		C					T		G	C	C		C	G
		Rangifer				G	T			A			C	A	A		C					T		G	C	C		C	G
	Cervinae	*Cervus elaphus*				G	T	A	G				C	A	A		C					T		G	C	C			G
		Cervus nippon				G	T	A	G				C	A	A		C					T		G	C	C			G
		Cervus albirostris				G	T	A	G				?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?
D	Camelinae	*Llama*				G	T						?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?	?

[1] The substitution at site 221 for bison could not be confirmed as it fell within the priming region.

Table 7-3. The 5 N terminal sequences of the SRY gene for eleven species of mammals are shown, where the underlined sequences indicate regions of the primers. Bold type indicates the insertion sequence in moose that ends in a second priming site.

	-120
Bos taurus (domestic cattle)	TATGTTCAGA CTATTGAACG ACGATGTTTA CAGTCCAGCT
Bison bonasus (wisent)	. . . . .
Bison bison (bison)	NNNNNNNNNN NNNNN..... . . . .
Ovis aries (domestic sheep)	. . . . . .A. . . . . .G
Gazella dorcas (gazelle)	. . . . . .T.... .A. . . . . .G
Capra hirta (domestic goat)	. . . . . .A. . . . . .G
A. americana (pronghorn)	NNNNNNNNNN NNNNN..... . . . . .TG
C. elaphus (wapiti/red deer)	NNNNNNNNNN NNNNN..... C
Rangifer tarandus (caribou)	NNNNNNNNNN NNNNN..... . . . .A....C
O. virginianus (white-tailed)	NNNNNNNNNN NNNNN..... . . . .A....C
Alces alces (moose)	NNNNNNNNNN NNNNN..... C

					-60
GTGGTACAGC	AACAAACTAC	TCTCGCTTTT	AGGAAAGACT	CTTCCTTGTG	CACAGACAGT
. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
. . . . .	. . . . .	....T.....	. . . . .	. . . . .	. . . . .
. . . . .	......A...	.T....C...	G.....AC..	. . . . .	. . . . . A.
. . . . .	......A...	......C...	G.....AC..	. . . . .	. . . . .
. . . . .	......A...	.T....C...	G.....AC..	. . . . .	. . . . . A.
. . . . .	...G..A...	......C...	G......C..	 C	. . . . . A.
.A.A....A.	......A.C.	......C...	G......C..	. . . . . T	. . . . . A.
AA.A....A.	......A.C.	......C...	G......C..	. . . . . T	. . . . . A.
AA.A....A.	......A.C.	......C...	G......C..	. . . . . T	. . . . . A.
.A.A....A.	.G....A.C.	......C...	G......C..	. . . . . T	. . . . . A.

						0
CATAGCGCAA	ATGATCAGTG	TGAAAGGGGA	GAACATGTTA	GGGAGAGCAG	CCAGGACCAC	
. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	T
. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	T
. . . . .	.C.....C.	. . . . . G	...A.....	. . . . .	. . . . .	
. . . . .	. . . . .	. . . . .	...A.....	. . . . .	. . . . .	
.....T....	. . . . .	 C	...A.....	C.	. . . . .	
.GC.....	. . . . .	.....C....	...A.C....	. . . . . GT	. . . . .	
.GC.....	. . . . .	.....CT...	...A.....	. . . . . G.	. . . . .	
.GC.....	. . . . .	.....C....	...A.....	....C...G.	. . . . .	
.GC.....	. . . . .	.....C....	...A.....	....C...G.	. . . . .	
.GC.....G	. . . . .	.....C....	...A....C.	C...C...G.	. . . . .	

GTGAAGCGAC	CCATGAACG
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
NNNNNNNNNN	NNNNNNNNN
. . . . .	. . . . . **GAGCCTGGTGGGCTACTGTCCACGGGGTCACAAAGAGTCA>**

>GGACATGAGTGAAGCGACCATGAACG